

Exploration of clay minerals in mitigating fish-killing algal blooms

By

Andreas Seger

B. Mar. Sc. (Hons), University of Tasmania

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Bluefin tuna mortality from a *Chattonella marina* bloom event in 1996 in Port Lincoln, South Australia (Courtesy: the late Barry Munday, UTAS School of Aquaculture)

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December, 2016

Statement of authorship (part of chapter 1)

The following people contributed to the publication of the work undertaken as part of this thesis:

Juan J. Dorantes-Aranda (40%, author 1), Andreas Seger (20%, candidate), Jorge I. Mardones (10%, author 3), Peter D. Nichols (10%, author 4) and Gustaaf Hallegraeff (20%, author 5).

Details of the authors roles:

J. J. Dorantes-Aranda and Gustaaf M. Hallegraeff contributed to the idea, development, refinement, presentation and formalization.

All authors contributed to the development, refinement, presentation and formalization.

J. J. Dorantes-Aranda, A. Seger (the candidate) and J. I. Mardones conducted the experimental work.

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published peer-reviewed manuscript contributing to this thesis:

Signed:

Professor Gustaaf Hallegraeff
Supervisor
Institute for Marine and
Antarctic Studies
University of Tasmania

Professor Richard Coleman
Executive director
Institute for Marine and Antarctic
Studies
University of Tasmania

Date: 1/12/2016

Statement of authorship (chapter 2)

The following people contributed to the publication of the work undertaken as part of this thesis:

Andreas Seger (20%, candidate) Juan J. Dorantes-Aranda (40%, author 1), Jorge I. Mardones (10%, author 3), Peter D. Nichols (10%, author 4) and Gustaaf Hallegraeff (20%, author 5).

Andreas Seger (50%, the candidate), Juan J. Dorantes-Aranda (15%, author 2), Alan R. Place (5%, author 3), Marius N. Müller (5%, author 4), Tae-Gyu Park (5%, author 5) and Gustaaf M. Hallegraeff (20%, author 6).

Details of the authors roles:

A. Seger (the candidate) and Gustaaf M. Hallegraeff contributed to the idea, development, refinement, presentation and formalization.

A. Seger (the candidate) conducted the experimental work.

All authors contributed to the development, refinement, presentation and formalization.

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published peer-reviewed manuscript contributing to this thesis:

Signed:

Professor Gustaaf Hallegraeff
Supervisor
Institute for Marine and
Antarctic Studies
University of Tasmania

Professor Richard Coleman
Executive director
Institute for Marine and Antarctic
Studies
University of Tasmania

Date: 1/12/2016

Statement of authorship (chapter 3)

The following people contributed to the publication of the work undertaken as part of this thesis:

Andreas Seger (50%, the candidate), Tae-Gyu Park (30%, author 2) and Gustaaf M. Hallegraeff (20%, author 3).

Details of the authors roles:

All authors contributed to the idea, development, refinement, presentation and formalization.

A. Seger (the candidate) and T. G. Park conducted the experimental work.

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published peer-reviewed manuscript contributing to this thesis:

Signed:

Professor Gustaaf Hallegraeff
Supervisor
Institute for Marine and
Antarctic Studies
University of Tasmania

Professor Richard Coleman
Executive director
Institute for Marine and Antarctic
Studies
University of Tasmania

Date: 1/12/2016

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Abstract

Fish-killing algal blooms increasingly threaten global aquaculture operations through the production of a wide variety of potent fish-killing toxins (ichthyotoxins). Effective mitigation strategies are therefore much needed. This thesis work explored the potential of clay minerals to not only remove harmful algal cells through flocculation, but instead focused on their ichthyotoxin adsorptive properties. Our experimental work explored 14 different clays (bentonite, kaolin, Korean loess, Phoslock™, zeolite), tested against 7 fish-killing algae (*Alexandrium* spp., *Chattonella marina*, *Cochlodinium polykrikoides*, *Heterosigma akashiwo*, *Karlodinium veneficum*, *Karenia mikimotoi*, *Prymnesium parvum*). Ichthyotoxicity was assessed with the gill cell line RTgill-W1 assay.

Extensive screening of different clays against the haptophyte *Prymnesium parvum* showed that removal of ichthyotoxicity was clay type and pH specific. At pH 9, only bentonite clays could completely remove ichthyotoxicity. However, other clays, such as Phoslock™ that worked well (57% removal) at pH 7, exacerbated ichthyotoxicity at pH 9 (by up to 30%), despite demonstrating high cell removal. We interpret this to be due to *Prymnesium* cell lysis caused by physical contact with clay, as well as pH induced changes in clay particle surface chemistry (e.g. zeta potential). Bentonite clay could completely eliminate *P. parvum*, *K. mikimotoi* and *K. veneficum* toxicity at clay loadings between 0.05-0.25 g L⁻¹, with detailed additional experiments revealing high swelling (<20 mL/2g) bentonites of fine particle size (<5µm) to be best suited.

While extracellular *C. marina*, *H. akashiwo* and *Alexandrium* spp. ichthyotoxins could only partially be removed, application of clay during cell lysis completely eliminated ichthyotoxicity. The likely reason appears to be the preferential adsorption of lipid peroxidation precursors (EPA, DHA) over highly toxic fatty acid aldehyde end products (deca- and heptadienal). *Chattonella marina* cell removal could be significantly improved through the addition of the flocculant polyaluminium chloride or synthetic amino-clays, which both proved excellent *C. marina* ichthyotoxin adsorbents. In the case of *A. catenella*, however, only bentonite clay proved suitable for ichthyotoxin removal purposes.

Finally, in August 2015, we worked with Korean National Institute of Fisheries Science scientists on routine clay dispersal operations targeting finfish farm threatening *Cochlodinium polykrikoides* blooms off Namhae Island (\$1.4M fish lost). Clay effectively reduced *Cochlodinium* cells to below levels considered dangerous to finfish aquaculture (<300 cells mL^{-1}), but exacerbated ichthyotoxicity by up to 32% compared to control sites. Simulated laboratory experiments with Korean loess confirmed that rapid (<5 min) *Cochlodinium* cell lysis after clay treatment caused increased ichthyotoxicity. Identical, finely ground (<30 μm) clay (not used in field) also generated significant cell lysis, but instead was capable of completely eliminating ichthyotoxicity. Laboratory manipulations with various Korean clay application regimes (pulsed additions, turbulence), indicated that only *Cochlodinium* concentration significantly influenced cell removal efficiency (higher removal at lower algal densities). Ground clay consistently outperformed conventional Korean loess (up to 20% higher cell removal). These findings highlight the potential of clay for ichthyotoxin adsorption, albeit HAB species, clay and pH specific, and point towards fine-tuning current practices for improved treatment efficacy.

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Chapter 1

Introduction | Rationale | Objectives

1. Introduction

1.1 Harmful algal blooms

The microscopic algae of the world's oceans are an essential component of the marine ecosystem, forming the basic trophic level on which the rest of the food-web relies. Rapid proliferation (so-termed blooms) of this group during favorable conditions may therefore be considered beneficial to fisheries productivity. However, blooms of certain microalgae, have been implicated in mass mortalities of cultured fin- and shellfish (Cembella et al., 2002; Imai et al., 2006; Park et al., 2013; Taylor and Trainer, 2002). The 60+ different algal species responsible for these deleterious effects are remarkably diverse (Cembella, 2003; Dorantes-Aranda et al., 2015; Rasmussen et al., 2016). While some algal species pose risks to cultured fish through indirect effects caused by the sheer number of algal cells (oxygen depletion or physical damage to fish gills), others are known to produce potent toxic compounds (Hallegraeff, 1993). The latter can be grouped into two broad categories: those that accumulate in shellfish and cause negative human health effects upon ingestion of contaminated seafood (true toxins of human health significance) and those that impact finfish (ichthyotoxins).

1.2 Threat to aquaculture operations

An apparent global increase in the occurrence of fish-killing algal blooms (HABs; Hallegraeff, 1993) combined with recent range extensions of harmful species (Hallegraeff, 2010; Kudela and Gobler, 2012; McLeod et al., 2012), has led to an increased exposure of a globally expanding aquaculture industry. Economic damages go into the millions of dollars (up to \$US400M for single events, Table 1.1) and the need to better understand bloom dynamics and fish-killing mechanisms to develop effective mitigation strategies has never been greater.

Table 1.1 Economic impact of single harmful algal bloom events by country and year of occurrence (listed by genus).

Causative genus	Country	Economic impact (\$US)	Year	Recurring Y/N	Reference
<i>Alexandrium</i>	Chile	60M	2002	Yes	pers. com. Hallegraeff (2013)
<i>Chattonella</i>	Japan	70M 50M	1972 2010	Yes	Taylor and Trainer (2002) Onitsuka et al. (2011)
	Australia	40M	1996	No	Hallegraeff et al. (1998)
<i>Chrysochromulina</i>	Norway	Cultured fish mortalities	1988	Yes	Simonsen and Moestrup (1997)
<i>Cochlodinium</i>	Japan	40M	2000	Yes	Itakura and Imai (2014)
	Korea	60M	1995	Yes	Park et al. (2013)
	Canada	2M	1999	Yes	Whyte et al. (2001)
<i>Heterosigma</i>	Japan	10M	1995	Yes	Itakura and Imai (2014)
	Canada	4-5M	1990	Yes	Horner et al. (1991)
	New Zealand	9M	1989	No	Chang et al. (1990)
<i>Karenia</i>	Japan	400M 13M	1984 2012	Yes	Itakura and Imai (2014)
	China	32M	1998	No	Yang and Hodgkiss (2004)
	United States of America	18M	1988	Yes	Hoagland et al. (2002)
	Australia (TAS)	3M	2003	No	de Salas et al. (2004)
<i>Karlodinium</i>	Australia (WA)	Wild fish mortalities	Annual	Yes	Place et al. (2012)
	United States of America				
<i>Noctiluca</i>	China	Cultured fish mortalities	1988	Yes	Huang and Qi (1997)

<i>Prymnesium</i>	Australia (NT)	20,000	2010	Yes	Body (2011)
	Norway	5M	1989	Yes	Johnsen et al. (2010); Kaartvedt et al. (1991)
	United States of America	318,000	2002	Yes	Tomas et al. (2002)

1.3 Summary of our current understanding of fish-killing algal blooms*

The species that have created highest impacts on fish farms worldwide are the unarmoured *Cochlodinium polykrikoides*, *Karenia mikimotoi* (dinoflagellates), *Chattonella marina*, *C. antiqua* and *Heterosigma akashiwo* (raphidophytes), *Prymnesium parvum* (haptophyte); more rarely the armoured dinoflagellates *Alexandrium catenella*, *A. tamarense* and the athecate *Karlodinium veneticum* have also caused adverse effects (Landsberg, 2002). Despite the impacts of these events, it remains to be clarified how these microalgae that do not produce chemically characterized toxins (different from neurotoxic, diarrhetic, amnesic and paralytic shellfish poisoning causing metabolites) are killing finfish.

Ichthyotoxicity has been attributed variously to production of free fatty acids (Fossat et al., 1999; Henrikson et al., 2010; Mooney et al., 2011) and reactive oxygen species or ROS (the superoxide radical O_2^- in particular) (Marshall et al., 2003; Oda et al., 1997) and occasionally to chemically defined phycotoxins such as brevetoxins or karlotoxins (Baden, 1989; Deeds et al., 2006; Van Deventer et al., 2012). However, not all ichthyotoxic microalgae produce these compounds in amounts that can account for their impacts on fish.

Reactive oxygen species are the result of electron transport, as occurs in metabolic processes within the cell. If molecular oxygen (O_2) accepts a single electron, the product is the superoxide radical; when O_2^- is reduced by a second electron, hydrogen peroxide (H_2O_2) is produced, and if reduction of H_2O_2 by a third electron occurs this can then lead to generation of hydroxyl radicals ($OH\bullet$) (Winston and Di Giulio, 1991). Reactive oxygen species are produced during respiration and photosynthesis, and can be significantly reduced using photosynthesis blockers, which suggests that fish mortality may be more prominent during day light hours (Marshall et al., 2002a).

Sensitive assays have been developed to quantify superoxide and hydroxyl radicals in aqueous solution. Both radicals have been detected in seawater; however, due to

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the high reactivity of hydroxyl, and thus its short lifetime ($\sim\mu\text{s}$) (Burns et al., 2012; Miller et al., 2011), only the superoxide anion can be accurately measured in cultures of microalgae (Godrant et al., 2009; Lee et al., 1995; Marshall et al., 2005; Oda et al., 1997). Superoxide lifetime in seawater has been measured in the range of 10-300 s (Burns et al., 2012). The improved MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3(7H)-one) assay adapted by Godrant et al. (2009) offers the advantage of performing superoxide tests on microplates, without having to use large volumes of algal cultures and hence enables better replication and also simultaneous measurements. This assay is based on the chemiluminescence of MCLA generated when it reacts with the superoxide radicals in the medium, as being produced by the algae. The signal is measured by a luminescence detector using a microplate reader. It should be noted that this technique quantifies the rate of O_2^- production after cell lysis (requires steady state signal) and not the total initial amount of O_2^- present.

Screening for generation of superoxide by a wide range of microalgae, has conclusively shown that raphidophytes of the genus *Chattonella* are the greatest O_2^- producers, generating up to 18 times more superoxide than other ichthyotoxic species, including raphidophytes and dinoflagellates (Marshall et al., 2005). There exists controversy in the ichthyotoxic mechanism of the dinoflagellate *Cochlodinium polykrikoides*, since early studies suggested the role of ROS but others found only trace levels, concluding that *C. marina* and *C. polykrikoides* have different toxic mechanisms (Kim et al., 1999; Kim and Oda, 2010; Tang and Gobler, 2009). Ichthyotoxic unarmoured microalgae are very fragile and susceptible to cell rupture; when this occurs, a cocktail of reactive compounds are released into the water. These compounds affect the fish mainly via gill damage during respiration, and this damage can be accelerated through hyperventilation under continuing stress conditions (Marshall et al., 2003). With some species, such as the armoured dinoflagellate *Karlodinium*, cell lysis has been conclusively demonstrated to be critical for ichthyotoxicity (Mooney et al., 2010), and similarly for the naked flagellate *Chattonella* more fragile strains are more potent fish killers (Dorantes-Aranda et al., 2013).

In previous experimental studies we presented a sensitive *in vitro* assay to test toxicity of harmful phytoplankton using a gill cell line from rainbow trout *Oncorhynchus mykiss* as a model (Dorantes-Aranda et al., 2013; Dorantes-Aranda et al., 2011). The premise of this approach is that fish gill damage is the first line of attack in harmful microalgae killing fish (Ishimatsu et al., 1996).

Application of this *in vitro* fish gill assay has allowed us to carry out sensitive screening tests for toxicity of living and lysed marine microalgae, while simultaneously measuring superoxide production. We confirmed that ichthyotoxic microalgae are generally more toxic after cell lysis (Fig. 1.1).

Gill cells showed lower viability upon exposure to lysed algal cells, except for the raphidophyte *Heterosigma akashiwo* (HAGB01), which was more toxic when cells were intact, possibly due to a highly labile chemical compound (Twiner et al., 2005). The armoured dinoflagellate *Karlodinium* is essentially nontoxic, except under conditions that cause cell lysis (Mooney et al., 2010). *Chattonella marina* was the most ichthyotoxic species, with no significant differences between Australian (CMPL01) and Japanese (N118) strains.

Chattonella marina was the greatest producer of the radical superoxide (Fig. 1.2), which confirms previous findings by Oda et al. (1997) and Marshall et al. (2005) that at least for this species, ROS are a key driver of ichthyotoxicity. Critically, our previous work showed that O_2^- produced artificially via the enzymatic system xanthine/xanthine oxidase (equivalent to *C. marina* levels), only affected gill cell viability by 14% (Mooney et al., 2011). This finding strongly suggests that ROS do not damage fish gill cells in their own right, but rather through synergistic reactions with other compounds, such as lipids through lipid peroxidation (Marshall et al., 2003).

In raphidophytes, ROS interactions with EPA can account for most of the ichthyotoxicity (Marshall et al., 2003), while our recent observations show that for *Alexandrium catenella*, ROS interactions with DHA play a key role (Mardones et al., 2015).

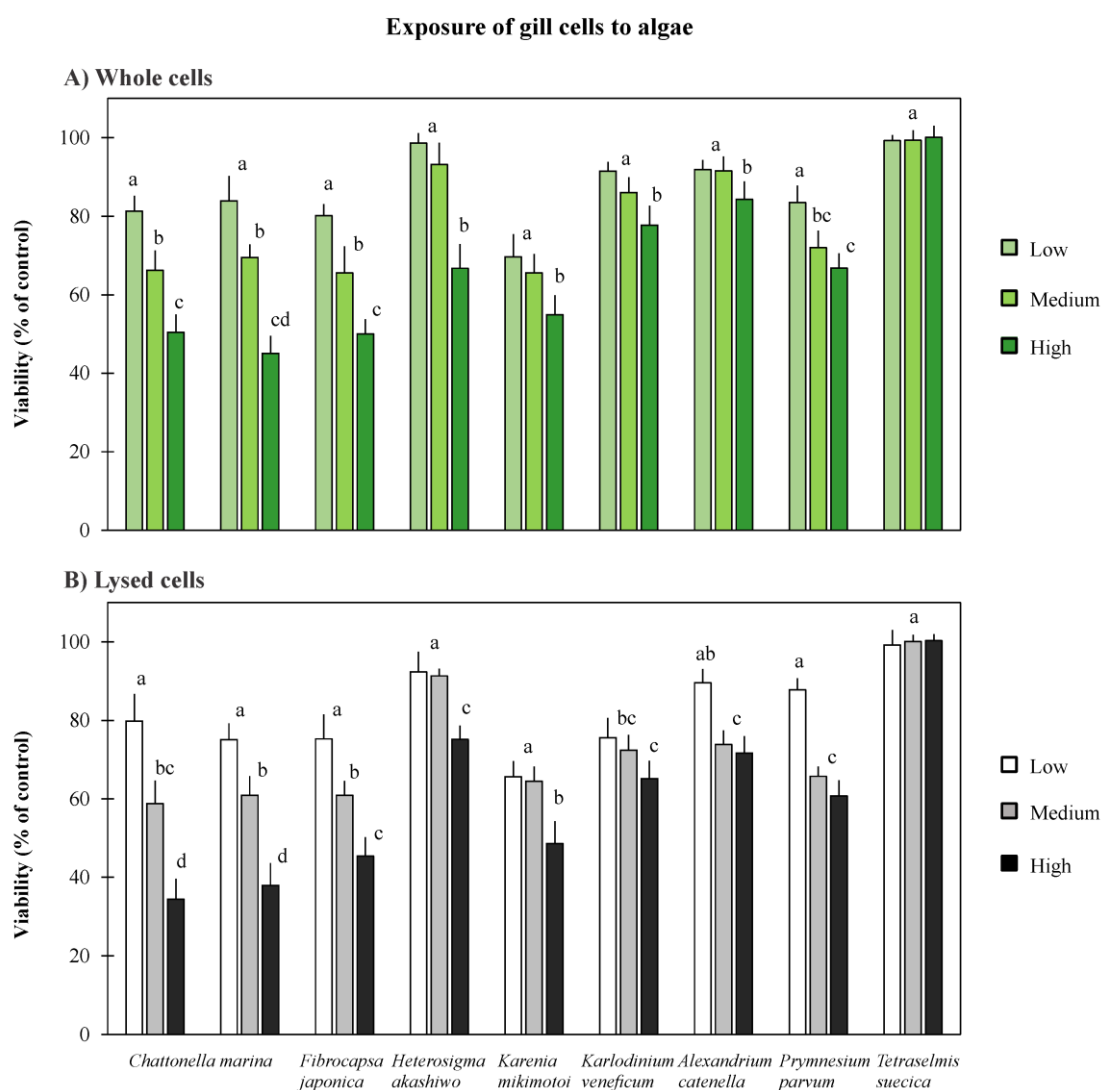


Figure 1.1 Viability of gill cells RTgill-W1 after a 2 h exposure to seven species of ichthyotoxic microalgae and one nontoxic. A) Whole algal cells, B) Lysed cells. Error bars indicate standard deviations, and letters on top of columns represent significant differences among treatments (whole and lysed cells of three concentrations) within each species. *Chattonella marina* includes the two strains CMPL01 (Australian) and N118 (Japanese). *Tetraselmis suecica* was used as a nontoxic control.

Among the products of lipid oxidation reactions that show adverse effects *in vivo* and *in vitro* are aldehydes, lipid radicals and lipid hydroperoxides (Jüttner, 2005; Pohnert et al., 2002). These compounds are highly reactive and able to attack cell

membranes and affect cell functions that may lead to death (Scandalios, 2002; Valavanidis et al., 2006). Our previous study showed toxicity by some aldehydes towards gill cells (Dorantes-Aranda et al., 2013); although the aldehydes tested were commercial preparations, aldehydes have been found in diatoms and haptophytes as a chemical defense against grazers (Hansen et al., 2004; Pohnert, 2000).

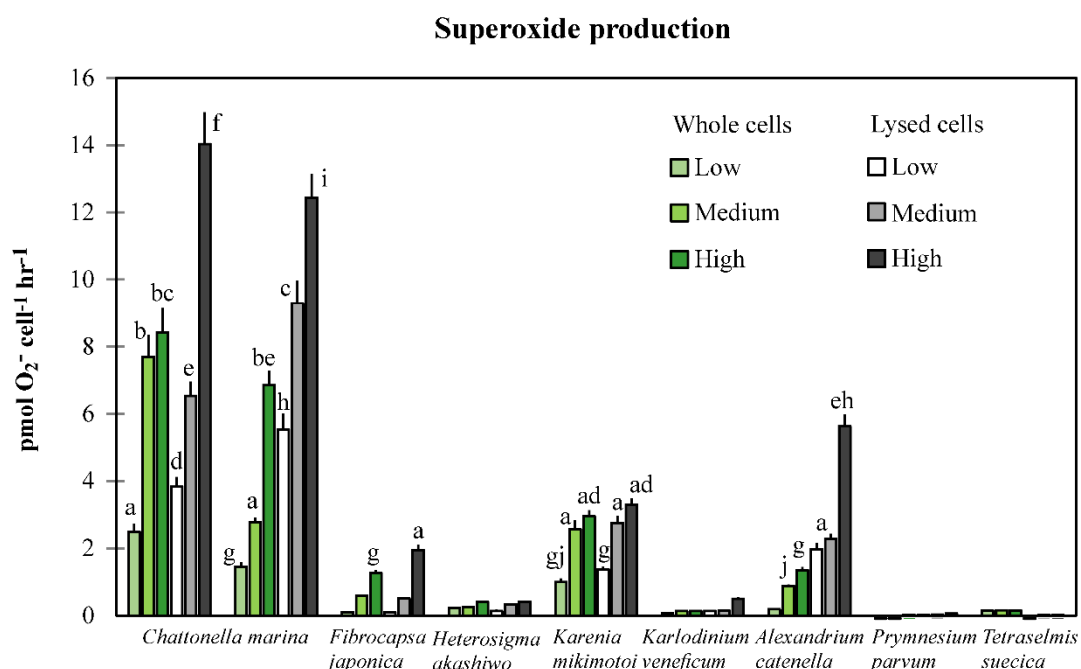


Figure 1.2 Production of superoxide radicals O_2^- by algal species under two conditions (whole and lysed cells) at three concentrations each. Error bars represent standard deviations of production rates ($n=3$), and letters on top of columns indicate significant differences among all treatments across species with production rates higher than $0.59 \text{ pmol cell}^{-1} \text{ h}^{-1}$. ANOVA, $F_{0.05;53,108}=441$.

It is clear that ichthyotoxic species do not always share common mechanisms. For instance, it is now well established that the dinoflagellate *Karlodinium veneficum* produces karlotoxins that affect gill cell viability (Place et al., 2012). The methanol extract of *K. veneficum* was the most toxic towards gill cells, and this result can quantitatively be attributed to the ichthyotoxicity of purified karlotoxin produced by this strain (i.e. all karlotoxin contained within methanol extract; Mooney et al., 2009). *Alexandrium catenella* produces a cocktail of hydrophilic PST toxins (neosaxitoxin and gonyautoxins, Aguilera-Belmonte et al., 2011), but purified PST

does not quantitatively account for ichthyotoxicity using our *in vitro* model since our recent experiments show that toxin concentrations equivalent to those produced by this alga affected gill cell viability by less than 30%, and the toxin concentrations used in the present study exceed the production levels observed in *A. catenella*. However, these observations still need to be confirmed with *in vivo* experiments. We observed that only aqueous, acetic acid and HCl extracts of *A. catenella* were toxic to gill cells (causing up to 65% decrease of gill cell viability), suggesting an important role of highly hydrophilic compounds. However, other lytic compounds are suspected to be the major culprit of *Alexandrium* (i.e. *A. tamarense*) mediated fish kills (Lush et al., 2001; Ma et al., 2009). Although their chemical nature and structure have not been fully described yet, it has been found that they are amphipathic compounds (with polar and non-polar components), extracted only with a mix of n-hexane and water (Ma et al., 2009).

The hypothesis of synergy between superoxide radicals and other ichthyotoxic compounds was first proposed by Marshall et al. (2003), showing that free fatty acids occurring together with superoxide accelerated fish mortality threefold. Our previous work showed that *C. marina* was able to produce high levels of free fatty acids (up to 33.2% of total lipids) and superoxide under conditions when rapid changes occurred, such as cell lysis and changes from dark to light conditions (simulating vertical migration, Dorantes-Aranda et al., 2013). Aqueous extracts of the raphidophytes *Chattonella marina*, *Fibrocapsa japonica*, and *Heterosigma akashiwo* did not show any effects on gill cell viability, but comparable methanol and acetone extracts were highly toxic, suggesting the role of hydrophobic compounds, such as fatty acids, in their toxic mechanism. Methanol is one of the solvents used for lipid extraction (Bligh and Dyer, 1959), and despite being a polar solvent, it is able to extract FFA to some extent. Hemolytic compounds have been purified from methanol extracts of *F. japonica*, and identified as PUFA (de Boer et al., 2009; Fu et al., 2004). Additionally, a fraction in 100% methanol purified from *Prymnesium parvum* was found to retain all the ichthyotoxic activity (Henrikson et al., 2010). It has been demonstrated that pH affects toxicity of fatty acid amides and fatty acids (Bertin et al., 2014). While early work reported that *Prymnesium*

ichthyotoxicity only occurred at pH ≥ 7.0 (Shilo and Rosenberger, 1960), the extent of toxicity did not increase between pH 7.5 and 9.0 (Shilo and Aschner, 1953). However, Ulitzur and Shilo (Ulitzur and Shilo, 1964) reported the opposite trend (increased toxicity at higher pH). Our recent observations during the exposure of gill cells to lysed *Prymnesium* at pH 7 and 9 did not detect any differences in toxicity. Interestingly, we did not observe any effect of pH (7.5 versus 9.0) on karlotoxin potency, showing that the role of pH in ichthyotoxicity is not universal.

Table 1.2 summarises the effects of live algae, algal extracts, toxins and other proposed toxic compounds that have been tested so far on the fish gill cell line RTgill-W1. Superoxide affected gill cell viability by only 14%; in contrast, purified karlotoxin KmTx-2 and brevetoxin PbTx-2 decreased gill cell viability by 99%. Fractions of free fatty acids extracted from *Chattonella marina* also decreased gill cell viability by 100%, as well as methanol and acetone crude extracts of the raphidophytes *C. marina*, *Heterosigma akashiwo*, *Fibrocapsa japonica*, and the haptophyte *Prymnesium parvum*.

In conclusion, our results contribute towards increasing the overall understanding of ichthyotoxicity of the microalgal species included in this study. Reactive oxygen species play an important role only with *Chattonella marina*; these ROS may also cause a synergistic effect with the lipids in the alga, producing other toxic compounds through lipid peroxidation (i.e. aldehydes and lipid radicals), which can cause physiological changes in gill cells by increasing superoxide dismutase activity, and damaging gill cell membranes. Karlotoxin accounts for toxicity by *Karlodinium veneficum*, and brevetoxins for *Karenia brevis* (but not *K. mikimotoi*); however, PST toxins are not the only toxic component in *Alexandrium catenella*. Other unknown compounds are involved in ichthyotoxicity by *Prymnesium parvum*, *Heterosigma akashiwo*, *Fibrocapsa japonica*, *Alexandrium catenella* and *Chattonella marina*, some of which clearly have a lipid component. Algal cell lysis of fragile fish killing HAB species is critical for ichthyotoxicity to occur, and environmental and physiological conditions (e.g. osmotic shock, turbulence, algal senescence, nutrient deficiency) that enhance this process deserve more attention in defining high alert situations for finfish aquaculture monitoring programmes.

Table 1.2 Summary of impacts of algae or toxins and substances produced by ichthyotoxic phytoplankton on viability of gill cells (RTgill-W1).

Alga, toxin or chemical	Source	Concentration	Decrease in gill cell viability (%)	Reference
<i>Chattonella marina</i>	Live culture, strain CMPL01	7×10 ⁶ cells L ⁻¹	55-71%	Dorantes-Aranda et al. (2011)
Superoxide radicals (O ₂ ⁻)	Xanthine/Xanthine oxidase	5-25 µM Xanthine + 30 U L ⁻¹ Xanthine oxidase	≤14%	Mooney et al. (2011)
Eicosapentaenoic acid (EPA)	Commercial preparation (METRAYA)	0.02-120 mg L ⁻¹	44-98.5%	Mooney et al. (2011)
Free fatty acid fractions	Extracted from <i>Chattonella marina</i>	0.44 µg mL ⁻¹	50%	Dorantes-Aranda et al. (2013)
		>3.17 µg mL ⁻¹	100%	
<i>Karlodinium veneficum</i>	Live culture, strain KVSRO1	1×10 ⁸ cells L ⁻¹	24-38%	Dorantes-Aranda et al. (2011)
OPA-rich MGDG ⁺	Extracted from <i>Karlodinium veneficum</i>	0.02-120 mg L ⁻¹	0-45%	Mooney et al. (2011)
OTA-rich MGDG ⁺⁺	Extracted from <i>Amphidinium carterae</i>	0.02-120 mg L ⁻¹	17-37%	Mooney et al. (2011)
Karlotoxin (KmTx-2) [^]	Purified from <i>Karlodinium veneficum</i>	0.1-1000 ng mL ⁻¹	3-99%	Dorantes-Aranda et al. (2015); Place et al. (2012)
2E,4E-decadienal	Commercial preparation (SIGMA)	0.34 µg mL ⁻¹	50%	Dorantes-Aranda et al. (2013)
2E,4E-heptadienal	Commercial preparation (SIGMA)	0.36 µg mL ⁻¹	50%	Dorantes-Aranda et al. (2013)
<i>Alexandrium catenella</i> extracts	Strain ACCH05 extracted in:			Dorantes-Aranda et al. (2015)
	Water	0.2-2.2% v/v	7-42%	
	Acetic acid 30 mM	=	2-43%	
	Hydrochloric acid 1 mM	=	2-65%	
PST toxins	National Research Council Canada			Dorantes-Aranda et al. (2015)
GTX1&4	Purified from <i>Alexandrium minutum</i> Strain AL1V	0.05-2.48 µg mL ⁻¹ GTX1 0.02-0.81 µg mL ⁻¹ GTX4	1-99.6%	

C1&C2	Purified from <i>Alexandrium tamarense</i> Strain AL18b	1.08-5.40 µg mL ⁻¹ C1 0.32-1.61 µg mL ⁻¹ C2	0-97.9%	
STX	=	0.50-2.47 µg mL ⁻¹	0.9-99.6%	
<u>Methanol extracts:</u>	Extracted in MeOH 99%			Dorantes- Aranda et al. (2015)
<i>Chattonella marina</i>	Au CMPL01	0.2-2.2% v/v	2-100%	
	Jp N-118	=	10-100%	
<i>Prymnesium parvum</i>	PPDW02	=	3-100%	
<i>Karlodinium veneficum</i>	KVSR01	=	6-100%	
<i>Heterosigma akashiwo</i>	HAGB01	=	5-100%	
<i>Fibrocapsa japonica</i>	FJCS332	=	34-100%	
<u>Acetone extracts:</u>	Extracted in Acetone 80%			Dorantes- Aranda et al. (2015)
<i>Chattonella marina</i>	Au CMPL01	0.2-2.2% v/v	0-100%	
	Jp N-118	=	0-100%	
<i>Prymnesium parvum</i>	PPDW02	=	6-100%	
<i>Karlodinium veneficum</i>	KVSR01	=	4-100%	
<i>Heterosigma akashiwo</i>	HAGB01	=	9-100%	
<i>Fibrocapsa japonica</i>	FJCS332	=	9-100%	
Brevetoxin (PbTx-2)	Purified from <i>Karenia brevis</i> (MARBIONC, USA)	0.1-40 µg mL ⁻¹	19-99%	Dorantes- Aranda et al. (2015)
Brevetoxin (PbTx-3)	Semisynthetic derivate from <i>K. brevis</i> (MARBIONC, USA)	0.1-40 µg mL ⁻¹	12-55%	Dorantes- Aranda et al. (2015)

* Equivalent to production rates by *Chattonella marina* at 6×10⁶ cells L⁻¹.

**Containing myristic (14:0, 11%), palmitic (16:0, 25%), palmitoleic (16:1ω7c, 11%), octadecatetraenoic (18:4ω3, 16%), and eicosapentaenoic (20:5ω3, 16%) acids.

+Octadecapentaenoic acid (Monogalactosyl diglyceride) 73%, with palmitic acid 26%

++Octadecatetraenoic acid 49%, with eicosapentaenoic acid 49%, and docosahexaenoic acid 2%

^Provided by Allen R. Place

1.4 Emergency fish farm management

The global multi-million dollar impacts of algal blooms on the finfish aquaculture industry highlight the urgent requirement for effective management strategies.

Boesch et al. (1997) identify three major approaches to HAB management:

1. Prevention: Removal/limitation of ichthyotoxicity promoting factors
2. Control: Permanent eradication of harmful algal species from the system
3. Mitigation: Minimisation of ichthyotoxic impacts through temporary measures

1.4.1 Prevention

Preventing harmful algal blooms from developing is the most desirable approach, yet open marine systems do not easily lend themselves to manipulation. The development of blooms in closed systems, such as onshore fish ponds or water reservoirs, however, can be achieved to some extent for certain algal species through the control of nutrient or freshwater input (Roelke et al., 2007, Kurten et al., 2010, Body, 2011, Hayden et al., 2012). A recently proposed, biological approach to prevention focuses on the role of sea grass beds (*Zostera marina*) as continuous seed banks of algicidal bacteria originating from surface biofilms on seagrasses and seaweeds (Imai, 2015). While the effectiveness of this approach remains to be investigated in detail in the field, it may prove suitable for localised, shallow water aquaculture areas.

1.4.2 Control

Aquaculture areas located in the coastal marine environment are subject to dynamic interaction between the terrestrial, atmospheric and marine domain. They are characterized by large scale connectivity between adjacent coastal regions and the open ocean. Algal blooms often develop offshore and are transported into coastal regions through currents, a process facilitated by a combination of up- and downwelling events (e.g. McGillicuddy et al., 2003). Increases in seasonal current strength and duration can greatly contribute to the establishment of HABs in regions from which they were previously absent or only seasonally present (Hallegraeff, 2010, McLeod et al., 2012), thereby increasing risks for aquaculture operations. This continuous and largely unpredictable recruitment makes the

control (i.e. elimination) of HABs near impossible, and effective, quick response mitigation strategies are required to react to threatening blooms in an environmentally friendly, effective and economically viable way.

1.4.3 Mitigation

Several different strategies have been trialed to mitigate the ichthyotoxic effects of HABs and extensive reviews are available (Sengco and Anderson, 2004, Sager et al., 2007, Hagström et al., 2010, Park et al., 2013). Strategies currently being practised in fish farm waters include cessation of fish feeding, towing away of cages from affected areas, perimeter skirts to protect against algal surface slicks, aeration or airlift upwelling to dilute harmful algal concentrations, or clay flocculation to reduce numbers of harmful algal cells (Rensel and Whyte, 2003).

Early work focused on the treatment of developing blooms with algicidal chemicals, such as ammonium- or copper sulphate (Shilo and Shilo, 1953, Shilo, 1962). These chemical treatments risk cell lysis and amplification of ichthyotoxic effects through release of intracellular metabolites (Deeds et al., 2002) and their use has been discontinued due to high collateral damage and significant toxicity towards fish (Barkoh et al., 2004, Sager et al., 2007). An alternative approach proposes the biological control of harmful algal blooms with marine viruses and bacteria (Bai et al., 2013; Jeong et al., 2008; Nagasaki et al., 1999). While some authors report high taxonomic specificity towards the target algal species (even strain specificity, Nagasaki et al., 1999), others have observed a more broad spectrum of affected organisms (Bai et al., 2013). A related biological mitigation method is the introduction of protistan grazers to a developing bloom (Jeong et al., 2008). However, depending upon initial algal and grazer density, as well as consumption rate, it can take up to an estimated 9 days for a bloom to be consumed through grazing (Park et al., 2013). No field trials using these biological approaches have yet been conducted and further research is clearly required to ensure their safety and cost-effectiveness for large scale applications. The well-established rapid flocculation of harmful algal cells through clay dispersal (applied in Southeast Asia for more than two decades), appears to be the most promising currently available

mitigation strategy (Sengco and Anderson, 2004, Hagström et al., 2010, Park et al., 2013).

1.4.3.1 Clay flocculation

First pioneered by Shirota (1989) in Japan and Yu et al. (1994b) in China, clay dispersal aims to promote the generation of rapidly sinking clay-algal aggregates that entrain additional cells during their descent. In Korean waters, where this approach has been extensively practised for the last two decades, clay application has been claimed to reduce overall fisheries damages of yearly recurring *Cochlodinium polykrikoides* blooms by up to 80% (Park et al., 2013). Several laboratory experiments investigating the efficacy of this approach have reported excellent cell removal efficiencies (up to 100%), dependent upon clay type, target species, algal concentration, turbulence and clay loading (Archambault et al., 2003; Archambault et al., 2004; Beaulieu et al., 2005; Hagström and Granéli, 2005; Padilla et al., 2010; Park et al., 2013; Sengco and Anderson, 2004; Sengco et al., 2001; Sun and Choi, 2004; Yu et al., 1994a).

In Korean waters, 1000-97,000 tons of clay are applied each year (Park et al., 2013) and repeated concerns have been raised for benthic communities due to high loads of suspended particles and sedimentation of toxic algal detritus (Archambault et al., 2004; Beaulieu et al., 2005; Shumway et al., 2003). Lewis et al. (2003) and Haubois et al. (2007) demonstrated that clay flocculation of *Karenia brevis* cells did not increase toxicity to benthic organisms compared to the effect from an untreated bloom. No negative effects of clay particles on fish themselves have been reported, which display irritated coughing behavior immediately after clay application, but soon recover as the clay settles (Rensel and Anderson, 2002).

Sengco (2001) screened 25 American clays and determined that higher removal efficiencies against *Karenia brevis* and *Aureococcus anophagefferrens* could only be achieved with Florida phosphatic bentonites and not zeolite or kaolin clays.

However, in removal experiments with the dinoflagellates *Noctiluca scintillans*, *Prorocentrum minutum* and the diatom *Skeletonema costatum*, Yu et al. (1995) found that kaolin was much more effective than montmorillonite (approx. 40% higher). There is currently no comprehensive explanation for these differences.

Each clay-algal system appears to be unique, with certain clay types being better suited for the removal of specific algal species.

As per strict geological definition, clay minerals are differentiated by their distinctive physicochemical properties. In practice, however, naturally mined products often present a conglomeration of different clay materials and/or other impurities, such as organic material (Haynes and Naidu, 1998). This complicates selection of suitable clay products and necessitates extensive, algal species specific screening (see Park et al., 2013; Sengco et al., 2001). These and subsequent studies (Hagström and Granéli, 2005; Sengco et al., 2005; Yu et al., 1994b, 1994a) revealed that factors such as pH, cation exchange capacity (cations are not 'fixed' to the outer surface of clays and can be exchanged) and the physical condition of algal cells (nutritional status) can significantly influence cell removal.

Another important factor to consider is cell size and cell surface properties (Sengco et al., 2001). Larger *Chattonella marina* cells (50 µm) were not readily sedimented (<20% removal, Wu et al., 2010) and armoured dinoflagellates, such as *Alexandrium tamarense*, required higher clay loadings (up to 10 g L⁻¹) for effective flocculation to occur (Sengco et al., 2001; Sun and Choi, 2004). However, modification of clays with surfactants (Gemini, sophorolipid) or flocculants (polyaluminium chloride) can greatly increase cell removal and lower required clay concentrations by up to an order of magnitude (Pierce et al., 2004; Sengco and Anderson, 2004; Sengco et al., 2001; Wu et al., 2010).

1.4.3.2 Ichthyotoxin removal

Although the main mechanism of cell removal appears to be flocculation, some authors report cell lysis after clay application due to physicochemical interactions between clay particles and algal cells (Archambault et al., 2003; Lee et al., 2013b; Rivera, 2014, October; Sengco, 2001; Shirota, 1989). Cell lysis can greatly amplify fish-killing effects (Deeds et al., 2002; Dorantes-Aranda et al., 2015; Dorantes-Aranda et al., 2011), indicating that under certain conditions, clay treatment may inadvertently increase ichthyotoxicity. However, recent research suggests that thus released ichthyotoxins could be directly adsorbed onto clay particles. Brevetoxins, cyanobacterial microcystins and haemolytic activity of *Prymnesium parvum*

ichthyotoxins could be completely removed or significantly reduced through treatment with bentonite clay (Morris et al., 2000; Pierce et al., 2004; Prochazka et al., 2013; Sengco et al., 2001).

The adsorption of toxic compounds to clay minerals is not a novel observation. In the agricultural sector, clay adsorption of the commonly used herbicide bt (extracted from *Bacillus thuringiensis*) has been well documented (Helassa et al., 2011; Ozcan et al., 2012) and it has become common practice to add clays to stock feeds of cattle grazing in pastures where they might be exposed to fungal aflatoxins (Phillips, 1999). Extensive investigations into the fate of antibiotics in soils revealed complex interactions between intrinsic clay properties, such as cation exchange capacity, surface charge and density, swelling capacity, surface area, as well as charge, size, and structure of the adsorbate (Gao and Pedersen, 2005; Henrichs and Sugai, 1993; Jahed et al., 2014; Moyo et al., 2014; Polati et al., 2006; Shen, 2002). Interpretation of adsorption behaviour onto different clay materials in these studies was greatly facilitated by the known chemical structure of the compounds studied.

The unknown nature and complex mechanism of action of most ichthyotoxins, paired with the remarkable physicochemical diversity of clay minerals, provides a formidable challenge to the study of ichthyotoxin adsorption. Each clay-algal system will need to be addressed individually to fully explore the potential of clay to mitigate fish-killing effects.

2. Rationale

Apparent global increases in the occurrence of fish-killing algal blooms pose an unprecedented threat to globally expanding aquaculture operations and the development of cost-effective mitigation strategies has thus become imperative. Application of clay promises effective reduction of harmful algal cell numbers, yet can potentially amplify fish-killing effects by promoting algal cell lysis. Adsorption of thus released ichthyotoxins to clay minerals has previously only been demonstrated for bentonite clay and a few, select algal species. Anecdotal evidence from the agricultural sector predicts algal species and clay type specific ichthyotoxin adsorption.

3. Objectives

This thesis work assessed the ichthyotoxin adsorptive properties of clay minerals for all major fish-killing algal species to offer recommendations for fine-tuning of existing clay dispersal protocols through:

1. Assessment of clay application efficacy for onshore fish ponds (*Prymnesium parvum*).
2. Field studies in Korean waters determining suitability of current clay dispersal practices (*Cochlodinium polykrikoides*).
3. Assessment of extracellular ichthyotoxin adsorption (*Karlodinium veneficum* and *Karenia mikimotoi*).
4. Application of clay to reactive oxygen species producing raphidophyte microalgae (*Chattonella marina*, *Heterosigma akashiwo*, *Fibrocapsa japonica*).
5. Application of clay to armoured dinoflagellates (*Alexandrium catenella*, *Alexandrium tamarense*, *A. minutum*).

Each objective represents an experimental chapter fine-tuning the application of clay to each specific algal species/ichthyotoxic mechanism. In each chapter, the most effective clay type and minimal effective dosage were determined to be summarised in the final chapter (7). Recommendations for fine-tuning of existing clay application strategies are presented.

Chapter 2 *

Mitigating fish-killing *Prymnesium parvum* algal blooms in aquaculture ponds with clay: the importance of pH and clay type

Abstract

When a barramundi farm in tropical Australia suffered substantial fish mortalities due to a bloom of the ichthyotoxic haptophyte *Prymnesium parvum*, the farm manager decided to manipulate pond water N:P ratios through removal of phosphorus by addition of lanthanum-modified bentonite clay (Phoslock™) to successfully mitigate ichthyotoxic effects. We conducted *Prymnesium* culture experiments under a range of N:P ratios, screening 14 different clays (2 zeolites, 4 kaolins, 6 bentonites and 2 types of Korean loess) at pH 7 and 9 for cell flocculation and removal of ichthyotoxicity assessed with the RTgill-W1 cell line assay. Application of Phoslock™ to cultures grown at different N:P effectively removed 60%–100% of water soluble toxicity of live *Prymnesium* (dependent upon nutritional status). While most clays efficiently flocculated *Prymnesium* cells ($\geq 80\%$ removal), cell removal proved a poor predictor of ichthyotoxin adsorption. Extensive clay screening revealed that at elevated pH as commonly associated with dense algal blooms, most clays either exacerbated ichthyotoxicity or exhibited significantly reduced toxin adsorption. Interpretation of changes in clay zeta potential at pH 7 and 9 provided valuable insight into clay/ichthyotoxin interactions, yet further research is required to completely understand adsorption mechanisms. Bentonite type clays proved best suited for ichthyotoxin removal purposes (100% removal at

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ecologically relevant pH 9) and offer great potential for on-farm emergency response.

2.1 Introduction

The globally distributed haptophyte *Prymnesium parvum* has been identified as the causative organism behind several mass fish mortality events (Body, 2011; Johnsen et al., 2010; Roelke et al., 2011; Shilo and Shilo, 1953; Tomas et al., 2002). Blooms are characterized by their seasonal recurrence, rapid proliferation, wide salinity and temperature tolerance, as well as the production of highly potent ichthyotoxins, the chemical nature of which remains incompletely known (Henrikson et al., 2010; Manning and La Claire, 2010; Tomas et al., 2002). Recurring *Prymnesium parvum* blooms have become a routine problem for the Israeli *Tilapia* (Shilo and Aschner, 1953; Ulitzur and Shilo, 1964), the Texas striped bass (Roelke et al., 2011; Tomas et al., 2002) and more recently (June/July 2009) the barramundi (*Lates calcarifer*) pond-based aquaculture industry in tropical Australia (Body, 2011).

Prymnesium parvum blooms vary considerably in toxicity, from being weakly toxic during exponential growth in nutrient replete conditions (nitrogen:phosphorus 16:1), to generating fast, irreversible gill cell damage under nitrogen or phosphorus limitation (Hagström and Granéli, 2005; Johansson and Granéli, 1999; Seger et al., 2014). Due to the eutrophic nature of high throughput aquaculture ponds, these systems are deemed to be ecologically unstable and prone to disturbance. However, as semi-closed systems, they lend themselves to careful manipulation of nutrient ratios through either ammonium sulphate addition (Kurten et al., 2010; Kurten et al., 2007) or phosphorus removal by Phoslock™ clay (Body, 2011; Ross et al., 2008).

In 2009, after a *Prymnesium* bloom at an Australian barramundi farm resulted in complete pond mortality (AUS \$95,000 of fish lost), the manager of the Arda-Tek farm engaged in a preventative pond nutrient and pH manipulation experiment. An experimental pond was treated with Phoslock™ clay (30 kg / kg phosphate) and pH

adjusted to <7.7 by stimulating microbial growth through the addition of molasses (Body, 2011). Phoslock™, a bentonite clay, has been specially modified to irreversibly bind water dissolved phosphorus to lanthanum cations incorporated into its clay matrix, a strongly pH dependent process (Ross et al., 2008). After application, phytoplankton biomass became more stable, with reduced diurnal oxygen fluctuations and *Prymnesium* did not reoccur in the treated pond. By contrast, an untreated control pond developed a further *Prymnesium* bloom in June 2011 and again in March 2012 that killed AUS \$10,000 worth of mullet. During the initial fish-kill event in 2009, *Prymnesium parvum* (identified through light and transmission electron microscopy) was successfully isolated and established in culture for later experimental manipulation (PPDW02 strain).

The large economic and ecological damages incurred by *P. parvum* blooms across the globe provide a strong incentive to formulate effective mitigation strategies. Previous investigations have focused on the use of clay minerals as mitigation agents of *Prymnesium parvum* because of their ability to flocculate algal cells and thereby remove intracellular ichthyotoxins (Hagström and Granéli, 2005; Sengco et al., 2005). Later work, focusing on the clay mediated removal of *Karenia brevis* dinoflagellate cells and *Microcystis aeruginosa* cyanobacterial toxins, reported evidence of direct adsorption of water soluble, extracellular ichthyotoxins to bentonite type clays (Pierce et al., 2004; Prochazka et al., 2013). This latter property of clay deserves special attention, since algal cell lysis in response to collisions with clay particles has previously been reported (Lee et al., 2013b; Rivera, 2014, October; Sengco, 2001; Sengco et al., 2001); a process that could greatly amplify ichthyotoxic effects through the release of intracellular ichthyotoxins (Deeds et al., 2002; Dorantes-Aranda et al., 2011). The potential of clays to adsorb *P. parvum* ichthyotoxins and the factors governing adsorption remain largely unknown. From studies modelling the adsorption of pesticide and pharmaceutical compounds to clay minerals in terrestrial systems, it appears likely that the increased seawater pH encountered during *P. parvum* blooms could constitute a significant variable

influencing ichthyotoxin adsorption (Figueroa et al., 2004; Fukahori et al., 2011; Polati et al., 2006).

We conducted *Prymnesium* culture experiments under different N:P ratios while measuring toxicity using the RTgill-W1 cell line assay to interpret field observations. Phoslock™ clay was applied to both sonicated and live algal cultures to assess effectiveness in removing *P. parvum* cells and their toxic effects. A preliminary account of this work was presented at the 15th International Conference on Harmful Algae (Seger et al., 2014). Since then, we screened 14 individual clay minerals belonging to four major clay groups (bentonites, zeolites, kaolins and Korean loess) for cell and ichthyotoxin removal properties at pH 7 and 9 to determine whether a highly modified clay such as Phoslock™ is required for efficient pond treatment. The zeta potential (approximation of clay surface charge) of the individual clay minerals and their physical characteristics are discussed to facilitate the selection of local clays as an on farm emergency response tool.

2.2 Methods

2.2.1 Clay Materials

Twelve commercially available Australian clays and two types of Korean loess were obtained and sieved through a 62 µm mesh to achieve a comparable size fraction (see Table 2.1 for details).

2.2.2 Phoslock™ (B3) Application

2.2.2.1 Live cultures

Non-axenic *Prymnesium parvum* (UTAS culture code PPDW02, isolated from barramundi aquaculture ponds during a harmful algal bloom event, Northern Territory, Australia in June 2009 by G. Hallegraeff), was grown in 6 L conical glass flasks containing 3 L of artificial seawater medium (ASW, Kester et al., 1967) with a salinity of 35 and nitrogen:phosphorus ratios of GSe/2 nutrients (based on Blackburn et al., 2001) adjusted to 16:1 (nutrient replete; 400 µM NO₃⁻ and 25 µM PO₄³⁻), 24:1 (phosphorus deficient; 400 µM NO₃⁻ and 16.7 µM PO₄³⁻) and 8:1 (nitrogen deficient;

200 μM NO_3^- and 25 μM PO_4^{3-}). Cultures were maintained at 20 ± 1 °C and 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of light with a 12:12 h light:dark cycle. Algal growth was monitored by cell counts (Coulter Counter, Beckman Z1). When cultures grown in nutrient deficient media entered stationary phase, these cultures and exponentially growing cultures (assumed to be nutrient replete), were diluted with artificial seawater without added nutrients (Kester et al., 1967) 24 h prior to experimental manipulation to yield 10^5 cells mL^{-1} (final pH 7.7-8.3). Cultures were divided into 7 replicates of 400 mL per nutrient treatment (500 mL twist-top jars, Labserv) to be randomly allocated to control and clay treatment groups ($n=3$). One 400 mL replicate was reserved for preparation of lysed cultures (see below). Lanthanum modified bentonite (Phoslock™, B3) was applied to cultures drop-wise in slurry form (prepared with ASW), such that the addition of 5 mL of clay slurry yielded a final clay concentration of 1.5 g L^{-1} . Control cultures received 5 mL of ASW and controls for the effect of Phoslock™ consisted of clay treated culturing medium. Cell numbers were counted 4 h and 3 days after treatment with a Beckman Coulter Counter (Z1) and the identical subsamples (collected 5 cm below surface and 3 cm above bottom of the jars) tested on the gill cell line RTgill-W1 in semi-permeable inserts (see below). Particle counts from seawater plus Phoslock™ controls were subtracted from particle counts of Phoslock™ treated algal cultures to facilitate interpretation of results without added clay particle interference.

2.2.2.2 Lysed cultures

Prymnesium parvum cells in the remaining 400 mL replicate of each nutrient condition were lysed through sonication (Measuring & Scientific Equipment Ltd., London, England, probe diameter of 2.5 mm at 9 μm peak to peak, 10 min) and split into six replicates of 40 mL per nutrient treatment. Replicates were assigned to treatment and control groups as described for live cultures above ($n=3$) and 2 mL of Phoslock™ slurry applied to yield a final concentration of 1.5 g L^{-1} . Unfiltered subsamples were tested on the gill cells 4 and 24 h after clay treatment in semi-permeable inserts.

Table 2.1 Physical properties of different clay types as provided by manufacturer.

Clay group	Clay code	Major clay type	Product name/Company	Cation exchange capacity (CEC)	Suppliers description
Bentonite	B1	Ca-bentonite	B1-AM/Bentonite Products WA, Watheroo, Australia	N.a.	50% A, 50% Gypsum; low swelling
	B2	Ca-bentonite	Es/ Bentonite Products WA, Watheroo, Australia	95.70	39.02% clay, 9.44% coarse sand, 37.39% fine sand, <14.15% silt
	B3	Modified Na-bentonite	Phoslock™/Phoslock Water Solutions Ltd, Sydney, Australia	N.a.	Lanthanum-modified bentonite (5% La ³⁺)
	B4	Ca-bentonite	A/ Bentonite Products WA, Watheroo, Australia	64.54	81.9% clay, 2.2% coarse sand, 16% fine sand, <0.01% silt
	B5	Ca-bentonite	Ed/ Bentonite Products WA, Watheroo, Australia	85.80	83.4% clay, 4.4% coarse sand, 9.8% fine sand, <2.5% silt
	B6	Na-bentonite	Trubond/Sibelco, Sydney, Australia	82	Montmorillonite as major component
Kaolin	K1	Kaolin	Fine ceramic clay obtained from potter, Hobart, Australia	N.a.	N.a.
	K2	Kaolin	CA-1/Claypro, Junortoun, Australia	N.a.	Dry milled ball clay (kaolinite major component)
	K3	Kaolin	Kaolinite/Zeolite Australia, Werris Creek, Australia	29–39	60%–75% opaline silica, 20%–30% Kaolinite, 5% other clays and mica
	K4	Kaolin	Snobrite C/Sibelco, Sydney, Australia	N.a.	70%–95% kaolinite, 5%–15% quartz, <15% other minerals
Zeolites	Z1	Zeolite	Zeolite/Zeolite Australia, Werris Creek, Australia	>120	Clinoptotilite with minor amounts of quartz, semectite
	Z2	Zeolite	ANZ38/Castle Mountain Zeolites, Quirindi, Australia	147	85% Clinoptotilite, 15% Mordenite), minor amounts of quartz, feldspar, montmorillonite
Korean loess	Kor 1	Korea A	Sourced from Tongyeong, South Korea	N.a.	Korean loess
	Kor 2	Korea B	Sourced from Geoje, South Korea	N.a.	Korean loess

2.2.2.3 High pH culture

Prymnesium cultures were grown in GSe medium prepared from filtered coastal seawater (0.2 µm, Pall) with a salinity of 30 to test the efficacy of Phoslock™ to remove ichthyotoxins and algal cells when applied to high density *Prymnesium* cultures at higher pH. Cultures were harvested during the stationary growth phase (8.6×10^5 cells mL⁻¹, pH 9.8) and 30 mL aliquots transferred to six 40 mL borosilicate test tubes. Three replicates were treated with 2 mL of Phoslock™ slurry prepared in deionized water (DI; milliQ), with control cultures receiving 2 mL of DI. Four h after treatment, subsamples were taken just below the surface and 2 cm above the bottom of the tube to be tested on the gill cells in semi-permeable inserts. The particle size distribution (3-10 µm equivalent spherical diameter; ESD) in these identical samples was determined through Coulter Counter analysis (Beckman, Z4) and the removal efficiency of algal cells (RE) calculated (Eq. 1) as:

$$RE = \left[\left(1 - \frac{Count_{treated}}{Count_{control}} \right) * 100 \right]$$

To account for increased particle counts in clay treated samples towards the lower end of the size spectrum (<4 µm), a further three test-tubes received 30 mL of GSe medium and were treated with Phoslock™.

2.2.3 Clay screening

2.2.3.1 Cell removal

Thirteen different clay types (Table 2.1) were applied to *Prymnesium* cultures grown in standard GSe medium (N:P 10:1) and harvested during the early stationary phase to evaluate the suitability of different clay types for flocculation purposes. Cultures were diluted with 0.2 µm filtered coastal seawater to yield 10^5 cells mL⁻¹ and the different clays applied to 30 mL of culture in 40 mL test tubes as described above for Phoslock™. Four h after clay treatment, the entire culture supernatant was collected down to a depth of 0.5 cm above the clay pellet and gently mixed prior to measuring chlorophyll a fluorescence of duplicate 2 mL sub-samples in a plate reader at excitation and emission wavelengths of 450 and 485 nm, respectively (Fluostar Optima, BMG Labtech, Ortenberg, Germany). Fluorescent measurements

of these duplicate samples (pseudoreplicates) were averaged for each replicate within each treatment and the standard deviation calculated between true replicates (n=3). To eliminate background fluorescence, clay added to seawater was employed as a blank. Cell RE was calculated as described above (Section 2.2.3) by comparing fluorescence in clay treated and control samples. Clay type K4 (employed in later ichthyotoxin removal experiments) was not available to us at the time of cell removal screening.

2.2.3.2 Ichthyotoxin removal

To determine the capacity of different clay types to adsorb ichthyotoxins released by lysed *P. parvum* preparations towards the gill cells, fourteen different clay types (Table 2.1) were evaluated at pH 7 and 9. *Prymnesium parvum* was harvested during the late stationary growth phase and concentrated through centrifugation of 10 mL of culture (10 min, 3300 x g, 20 °C, Sorvall Super T21, Du Pont, Delaware, USA). The upper 9 mL were discarded and the remaining 1 mL immediately sonicated for 1 min on ice. Lysed, concentrated culture was diluted in L-15/ex medium (Schirmer et al., 1997) to yield a concentration equivalent to 10^5 cells mL⁻¹. The pH of the L-15/ex medium was adjusted to 7 and 9 with either 0.1 M HCl or NaOH prior to addition of lysed culture. It is acknowledged that the final pH would have slightly deviated upwards from the original pH of 7 and 9, due to the addition of clay slurry and culture lysate, but this was not quantified. Clay slurries were prepared, such that the addition of 0.5 mL of slurry to 5 mL of *P. parvum* preparation yielded a final clay concentration of 1.0 g L⁻¹. Toxic controls received an equivalent volume of DI, whereas non-toxic controls consisted of clay addition to pure L-15/ex medium. After treatment, samples were vortexed for 5 s and subsequently centrifuged (1400 x g, 5 min, Hettich Universal 16A, Tuttlingen, Germany). Without disturbing the clay pellet, 3 mL of supernatant were collected, mixed and tested on the gill cells in conventional 96-well plates.

2.2.3.3 Zeta potential

The zeta potential of different clay types was determined with a Nanoseries zetasizer Model Zen3600 (Malvern Instruments, Malvern, Worcestershire, UK). This

instrument calculates the surface charge of particles based on their electrophoretic mobility in an applied electric field. All measurements were conducted at 25 °C and a clay concentration of 1.0 g L⁻¹ in L-15/ex medium at pH 7 and 9 (refractive index 1.333, viscosity 9.18 × 10⁻⁴ Pa·s and dielectric constant 80.0). Smoluchowski approximation was applied due to large particle size (>1 µm) and high ionic strength (>100 mM Hunter, 1981). Briefly, the Smoluchowski approximation takes into account the effect of solutions with higher ionic strength on the particle's electrical double layer thickness and therefore their measured electrophoretic mobility to calculate the particles' surface charge.

2.2.3.4 Minimum effective dose

The minimum effective dose of Ed (B5) and Phoslock™ (B3) clay was determined during a simulated worst case scenario of complete cell lysis at higher pH likely to be encountered during a dense, natural *Prymnesium* bloom. Lysed preparations from high density cultures (1.2 × 10⁶ cells mL⁻¹) were prepared as described above and diluted in L-15/ex to yield 4 × 10⁵ cells mL⁻¹ (moderate bloom densities). The pH of the L-15/ex medium was adjusted to the original culture pH of 9.2 and salinity to 30 through NaCl addition. In Eppendorf tubes, 20 µL of clay slurry was added to 980 µL of lysed *P. parvum* preparation to yield a final clay concentration of 0–0.25 g L⁻¹. Samples were vortexed and centrifuged as described above, with the final centrifugation step conducted in a microcentrifuge (7800 x g, 1 min, Micromax, Thermo Electron Corporation, Milford, USA). The resulting supernatant was tested with the gill cell line assay in conventional 96-well plates.

2.2.4 Gill cell line assay

The gill cell line viability assays were conducted following the protocols described in detail in Dorantes-Aranda et al. (2011). This bioassay allows for the exposure of gill cells to lower salinity samples (<10) in conventional 96-well plates, or higher salinity samples in more specialized semi-permeable membrane systems (Corning 3381, Acton, USA). Briefly, for conventional plates, 100 µL of 2 × 10⁵ gill cells mL⁻¹ suspended in L-15 medium (L1518, Sigma) were seeded into 96-well plates (Greiner 665180, Kremsmünster, Germany) and allowed to attach for 3 days at 20 °C in the

dark. Prior to testing of samples, the L-15 medium was discarded and cells rinsed twice with 100 μ L phosphate buffer saline (PBS). Subsequently, 100 μ L of sample was added to each well and plates incubated in the dark for 3.5 h. After exposure, samples were discarded, rinsed twice with PBS and finally 100 μ L of 5% resazurin (Sigma, 199303, Castle Hill, Australia) in L-15/ex added. Plates were incubated in the dark for a further 2 h and gill cell viability quantified by measuring the metabolic reduction of the resazurin dye to fluorescent resorufin in a plate reader at excitation and emission wavelengths of 540 and 590 nm, respectively (Fluostar Optima, BMG Labtech, Ortenberg, Germany). Gill cell viability is reported as percentage of the respective non-toxic control.

2.2.5 Statistics

Model I, two-way analysis of variance (ANOVA) were conducted on the nutrient manipulation data (main effects nutrient status and treatment). Significant interactions of main effects were followed by post-hoc Tukey's HSD test. Where no significant interactions were observed, Welch's T-test was employed to test for treatment (Phoslock™) effect. To satisfy ANOVA assumptions of homogeneity of variance and normality, cell removal data was squared and gill viability data square root transformed. A significance level of 95% ($\alpha = 0.05$) was employed in all analysis, performed with the statistical package R (www.r-project.org). Where given in the text, mean values are reported as ± 1 standard deviation and error bars on figures represent the standard error ($n = 3$).

2.3 Results

2.3.1 Phoslock™ (B3) application

2.3.1.1 Live cultures

Application of Phoslock™ clay to live *Prymnesium parvum* cultures significantly reduced cell numbers across all nutrient conditions when compared to control cultures after 4 h (Fig. 2.1a). Dilution of experimental cultures 24 h prior to the experiment to 10^5 cells mL^{-1} did not negatively influence algal cells, as evident from continued exponential growth of cultures therefore assumed to be nutrient replete (1.5×10^5 cells mL^{-1} at start of experiment). Cell concentrations in cultures grown in

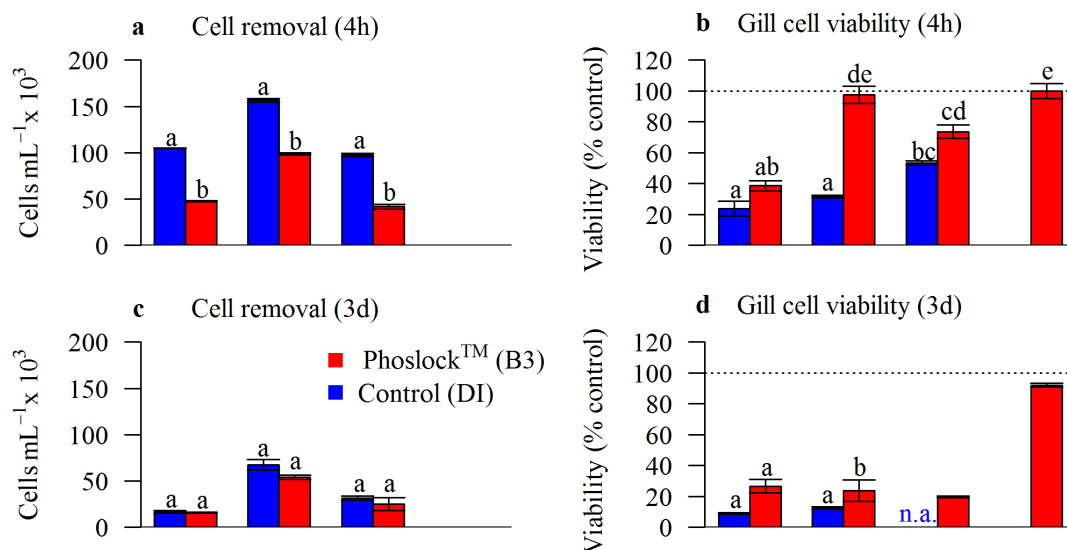
phosphorus and nitrogen deficient media remained stationary during the acclimation phase. While nutrient status and initial cell concentration did not influence the extent of cell removal ($5.7 \times 10^4 \pm 550$ cells mL⁻¹ removed across all nutrient treatments), they significantly influenced toxicity towards the gill cell line RTgill-W1 (Fig. 2.1b). Control cultures grown in nitrogen deficient media proved the most toxic towards the gill cells, followed by controls of the nutrient replete and phosphorus deficient treatments (with only 24 ± 8 , 32 ± 2 and $53 \pm 3\%$ of viable gill cells remaining, respectively). Four h after Phoslock™ application, no toxicity was detected in the nutrient replete cultures and gill viability increased by 15 and 21% compared to the untreated controls in the nitrogen and phosphorus deficient treatments, respectively (Fig. 2.1b). However, 3 days after clay treatment, toxicity was significantly increased in both controls and treated cultures across all nutrient conditions, although viability of gill cells exposed to Phoslock™ treated samples was still higher than for untreated controls in case of the nutrient replete treatment (Fig. 2.1d). Cell concentrations 3 days after clay application were significantly lower when compared to those 4 h after treatment, with no differences detected between cultures of the control and treatment groups within each nutrient condition (Fig. 2.1c).

2.3.1.2 Lysed Cultures

Lysis of algal cells through sonication significantly increased toxicity towards the gill cells, with only $13 \pm 2\%$ viable gill cells remaining in the nutrient replete treatment and no viable gill cells detected after exposure to control cultures of the nutrient deficient treatments (Fig. 2.1e). Significantly higher gill cell viability was detected in Phoslock™ treated samples, with viability increased to 20 ± 2 and $80 \pm 2\%$ in the nitrogen deficient and nutrient replete treatments, respectively. Gill cell viability in the phosphorus deficient treatment only increased marginally in response to clay addition ($+4 \pm 1\%$). Twenty four h after clay application, toxicity in control and clay treated cultures of the phosphorus deficient treatment was reduced (77 ± 2 and $89 \pm 5\%$ viability, respectively) and no toxicity detected in algal cultures of the nutrient replete or nitrogen limited nutrient treatments (Fig. 2.1f).

Phoslock™ (B3) application to *P. parvum* cultures grown at different N:P

Live Cultures



Lysed Cultures

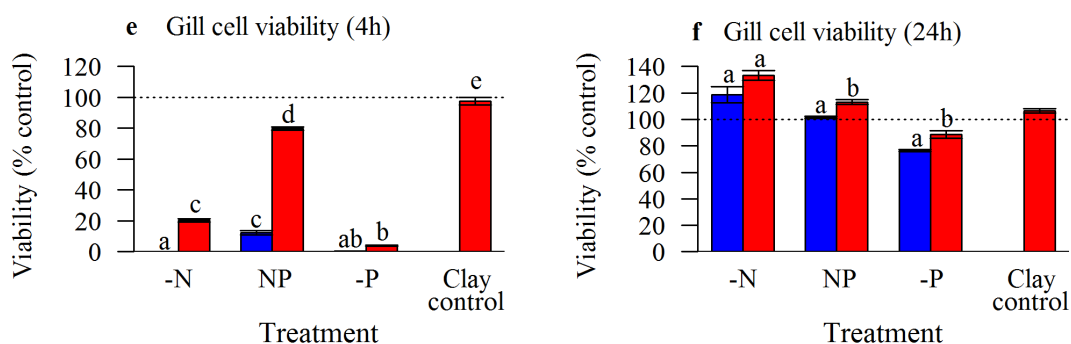


Figure 2.1 Lanthanum-modified bentonite clay (Phoslock™, B3) application to live and lysed *P. parvum* cultures grown under different nutrient conditions (nutrient replete, NP; nitrogen deficient, -N; phosphorus deficient, -P). Phoslock™ treated cultures received 1.5 g L⁻¹ of clay slurry (red bars) and controls an equivalent volume of deionized water (DI, blue bars). (a) Cell concentration and toxicity towards the gill cells (b) in live cultures 4 h after clay application. (c) Cell concentration in live cultures and toxicity towards the gill cell line RTgill-W1 (d) 3 days after clay treatment. (e) and (f) Toxicity of lysed *P. parvum* towards the gill cells 4 and 24 h after clay treatment. Letters represent significant differences between control and clay treatments within each nutrient condition (a,c,d and f; Welch's T-test) or between all treatment conditions (b and e; Tukey's HSD). N.a.: not available.

2.3.1.3 High pH culture

Phoslock™ (B3) application to high cell density (surface: 7.6×10^5 ; bottom: 9.8×10^5 cells mL⁻¹), high pH (pH 9.8), live *P. parvum* cultures did not significantly improve gill cell viability (Fig. 2.2a). The change in gill cell viability in response to Phoslock™ treatment was significantly influenced by sample location, as evident from a significant interaction effect between the main effects treatment and location ($F_{1,7} = 6.33$, $p = 0.04$).

Phoslock™ (B3) application to dense, high pH *Prymnesium* cultures

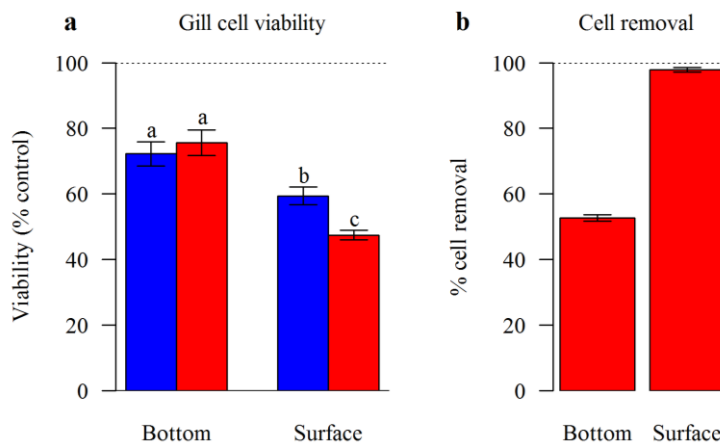


Figure 2.2 (a) Gill cell viability after exposure to high density (surface: 7.6×10^5 ; bottom: 9.8×10^5 cells mL⁻¹), high pH (9.8) *P. parvum* cultures treated with lanthanum-modified bentonite clay, Phoslock™ (red bars) and deionised water (control; blue bars). Letters indicate statistically significant treatment groups (Tukey's HSD). (b) Percentage cell removal efficiency in identical samples, collected 1 cm below surface and 2 cm above bottom of 40 mL test tube.

Viability of gill cells exposed to samples collected near the bottom of the test tube was reduced to an average of $74 \pm 6\%$, with no significant differences observed between control or clay treatments (Tukey's HSD: $p = 0.88$). Surface samples proved more toxic, with the control treatments reducing gill viability to $59 \pm 5\%$ and Phoslock™ application decreased gill cell viability to $48 \pm 3\%$, despite complete cell removal in these samples ($98 \pm 1\%$ RE; Fig. 2.2b). A cell removal efficiency of only $53 \pm 2\%$ was recorded in bottom samples. The particle size distribution in surface and bottom samples followed a normal distribution in the size range of 4–9 μm ESD

(equivalent spherical diameter) with a peak at 6 μm , corresponding to the *P. parvum* population (Fig. 2.3). In the lower size range ($<4 \mu\text{m}$), particle counts were considerably higher in the Phoslock™ treated samples (bottom: $(189 \pm 14) \times 10^3$, surface: $(126 \pm 11) \times 10^3$ particles mL^{-1}) than controls (bottom: $(65 \pm 10) \times 10^3$, surface: $(55 \pm 1) \times 10^3$).

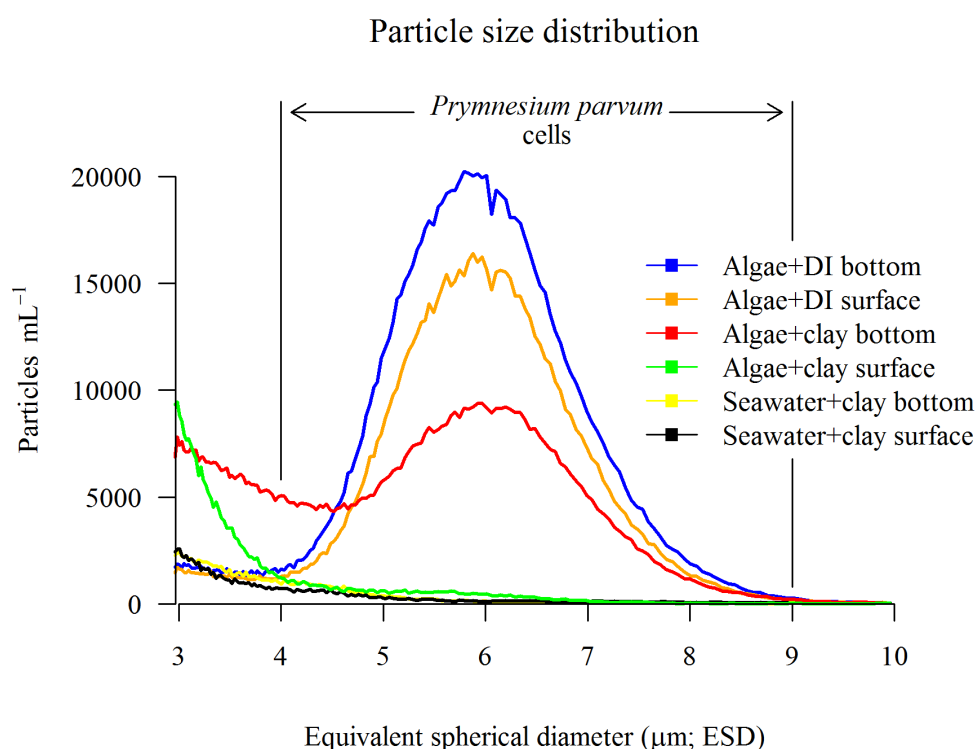


Figure 2.3 Particle size distribution in surface and bottom samples 4 h after Phoslock™ (B3) addition to *P. parvum* cultures. Cultures were treated with 1 g L^{-1} Phoslock™ slurry (black and orange lines represent top and bottom samples, respectively) and control cultures with an equal amount of deionized water (DI, red and blue lines represent top and bottom samples, respectively). To facilitate interpretation of results, a further control of 1 g L^{-1} Phoslock™ clay in seawater (no algae) was employed (yellow and black lines represent bottom and surface samples, respectively). The particle size range corresponding to *P. parvum* cells is indicated (4–9 μm ESD).

2.3.2 Clay screening for cell and ichthyotoxin removal

2.3.2.1. Cell removal

Prymnesium parvum cell removal efficiency (RE) differed significantly between individual clay types (ANOVA, $F_{12,26} = 29.03$, $p < 0.001$; Fig. 2.4). While no obvious trend in the clays' capacity to remove cells was observed between clay groups, most clays achieved high cell removal ($\geq 80\%$ RE). Only Korean yellow loess type B (Kor2) and the gypsum-bentonite composite B1-AM (B1) performed poorly (approx. 50% RE).

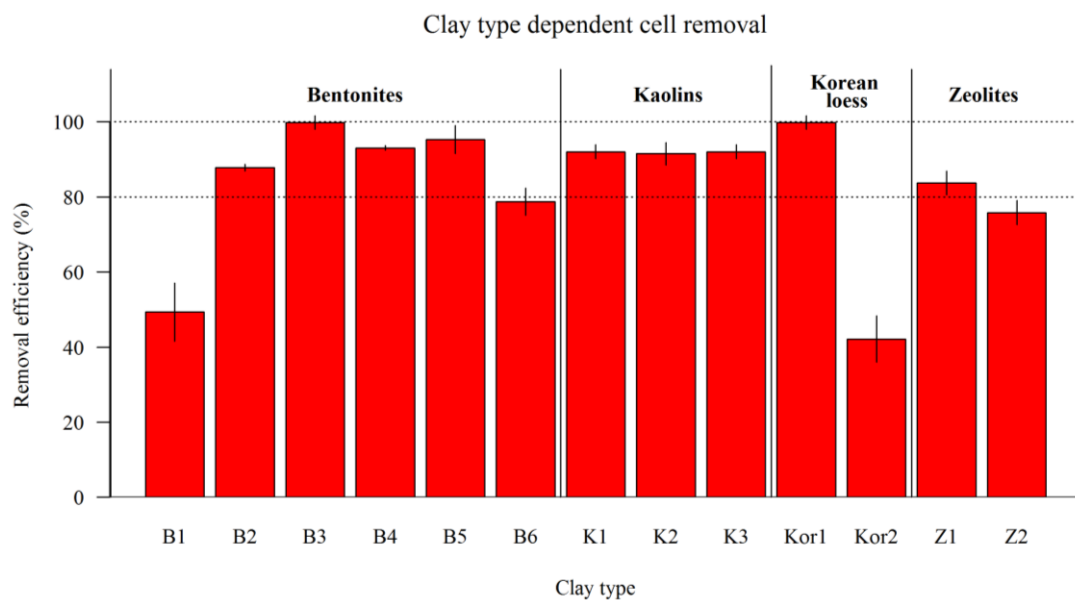


Figure 2.4 Removal efficiency of *P. parvum* cells by different clay types.

2.3.2.2 Ichthyotoxin removal

Significant differences between and within clay groups were detected when exposing gill cells to lysed *P. parvum* preparations at pH 7 and 9 (Fig. 2.5), with a two-way ANOVA indicating significant influence of both main effects and their interaction on gill cell viability (Table 2.2). While individual kaolins (K1-4), zeolites (Z1-2) and Korean loess (Kor1-2) behaved similarly within their clay group, large variations in the effect of the different bentonite types on gill cell viability were observed, particularly in response to pH. At pH 7, clay treatment generally increased gill cell viability, with the exception of the Korean loess group (Kor1-2) and the bentonite type B1 (no viability changes).

Table 2.2 Two-way analysis of variance (ANOVA) conducted on the main effects of clay type, pH and their interaction on gill cell viability. Degrees of freedom (Df), sum of squares (SS) and mean square values (MS) are provided.

Source	Df	SS	MS	F	P
Clay type	13	452.40	34.80	227.22	<0.001
pH	1	90.60	90.60	591.51	<0.001
Clay type * pH	13	141.00	10.85	70.83	<0.001
Residuals	56	8.60	0.15		

The largest improvements in gill viability relative to the toxic control at pH 7 were observed when ichthyotoxic preparations were treated with clays from the bentonite group (B2-6; average of +51%), followed by kaolins (K1-4; average of +26%) and zeolites (Z1-2; average of +22% gill viability). While no significant differences in the toxicity of controls were observed between pH 7 and 9 (1-way ANOVA: $F_{3,1} = 0.69$, $p = 0.468$), the influence of clay treatment on the gill cells differed significantly at these two pH values.

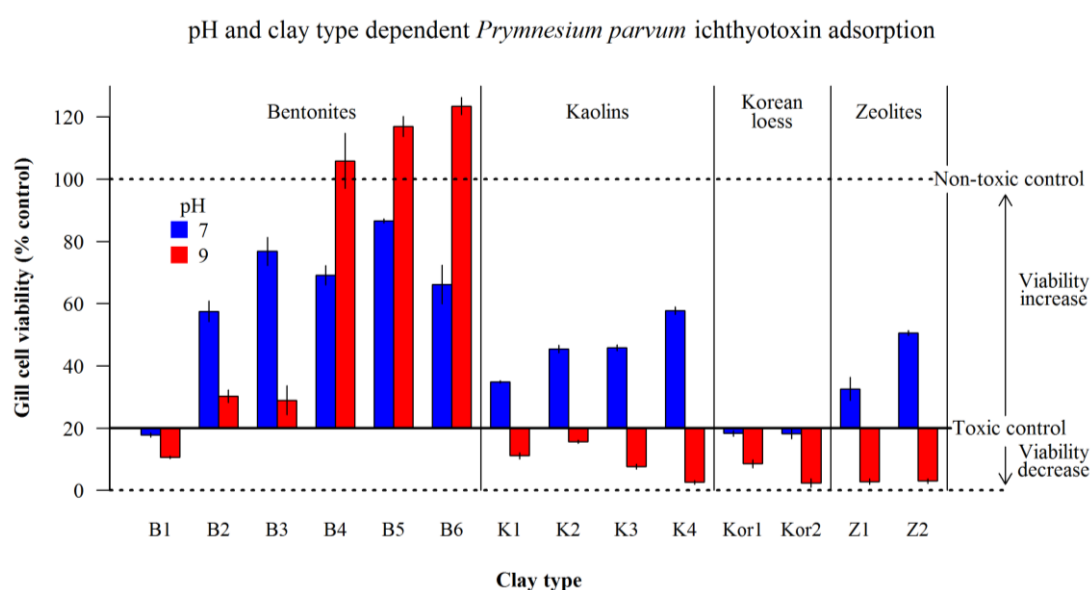


Figure 2.5 Gill cell viability after exposure to ichthyotoxic *P. parvum* preparations treated with different clay types at pH 7 (blue bars) and 9 (red bars). Arrows indicate direction of fish gill viability change compared to the toxic control (solid black line, 20% viability).

At pH 9, kaolins, zeolites and Korean loess exacerbated toxicity towards the gill cells, leading to complete viability loss in some cases (K4, Kor2, Z1-2). Similarly, bentonite type B2 (Es) and B3 (Phoslock™), which significantly improved gill viability at pH 7 (+38% and +57% gill cell viability increase, respectively), displayed only minor improvements in gill cell viability at pH 9 (+10% and +9%, respectively). Conversely, bentonite types B4-B6 performed best at pH 9, resulting in complete removal of toxicity towards the gill cells. Viability of gill cells exposed to toxic samples treated with B5 and B6 actually exceeded that of non-toxic controls (seawater + clay). This appears to be due to an interaction of clay and culture medium conditioned during algal growth as opposed to pure seawater (no algae); an observation only recorded for samples that had been rendered non-toxic through clay application.

Zeta potential measurements of clay suspensions revealed negative charges (> -15 mV) for all clay types (Fig. 2.6), with a two-way ANOVA indicating significant interactive effects of clay type and pH on zeta potential ($F_{13,29} = 11.44$, $p \leq 0.001$). Individual kaolins (K1-4), zeolites (Z1-2) and Korean loess (Kor1-2) exhibited similar zeta potentials within their groups and comparable effects of pH on the extent of that charge. At pH 7, kaolins (-33.9 ± 1.0 mV) displayed the largest negative potential, followed by zeolites (-30.5 ± 0.3) and Korean loess (-24.7 ± 0.4). At pH 9, the extent of the negative charge was reduced in all three groups when compared to pH 7 (kaolins: $+7.8$, zeolite: $+6.6$ and Korean loess: $+1.9$ mV). As previously observed for ichthyotoxin adsorption, the bentonite group showed non-coherent behavior at the two tested pH values. Similarly to the other clay groups, bentonite types B1-3 exhibited less negative charges at pH 9 when compared to pH 7, whereas the zeta potential of B4-5 (-25 ± 0.7 mV) remained unchanged and the negative charge of B6 increased (-34.1 to -38 mV at pH 9).

2.3.2.4 Minimum effective dose

Treatment of lysed *P. parvum* preparations (pH 9.2) with increasing concentrations of Phoslock™ (B3) and Ed (B5) type clays significantly increased gill cell viability relative to the toxic control ($41 \pm 7\%$ viability, Fig. 2.7). Ed clay treated preparations increased gill cell viability to $85 \pm 14\%$ at a clay loading of only 0.05 g L^{-1} , with no

viability loss observed at 0.25 g L⁻¹. Gill cell viability increased linearly with increasing Phoslock™ concentration, with the highest clay loading of 0.25 g L⁻¹ only increasing gill cell viability to 73±5% (compared to complete removal of toxicity by Ed clay at the same concentration).

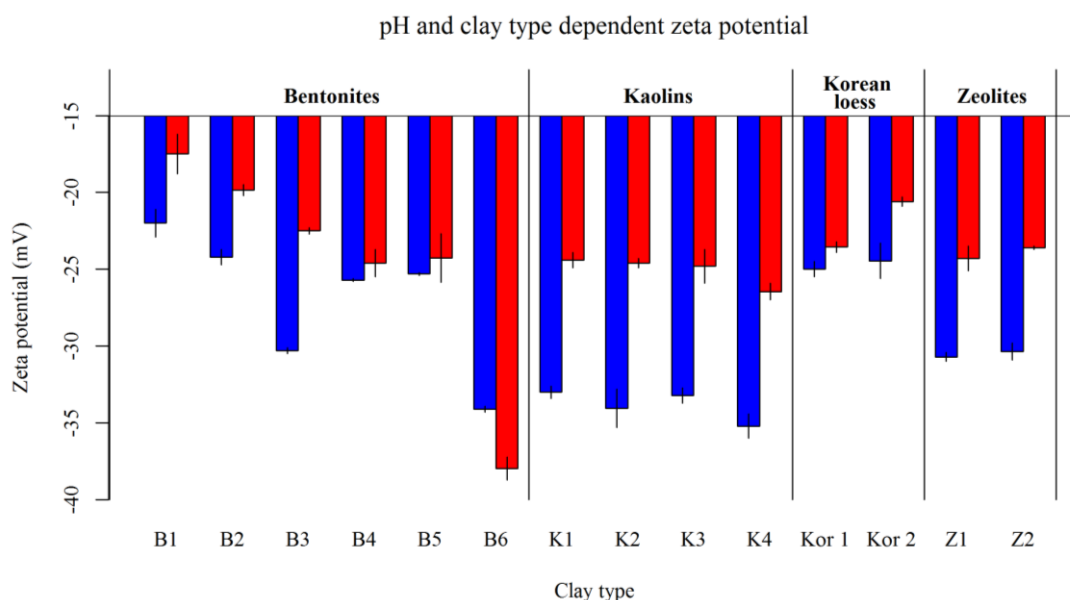


Figure 2.6 Zetapotential (mV) of different clay types in L-15/ex medium at pH 7 (blue bars) and pH 9 (red bars).

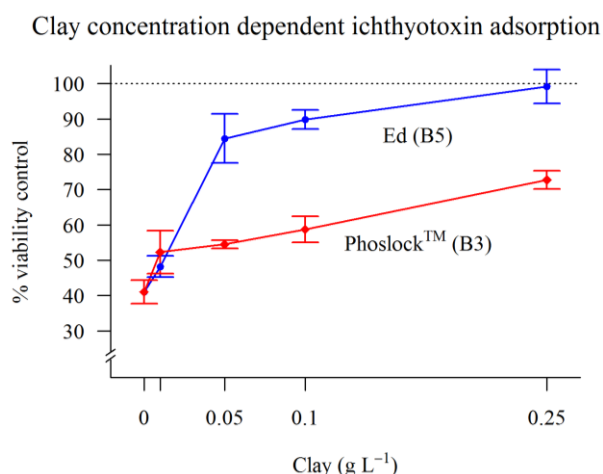


Figure 2.7 Gill cell viability after exposure to lysed *P. parvum* preparations treated with different concentrations (0–0.25 g L⁻¹) of Ed clay (blue circles) and lanthanum-modified bentonite (Phoslock™; red diamonds) at pH 9.2.

2.4 Discussion

2.4.1 Phoslock™ (B3) Application

Nutrient manipulation experiments, conducted to better understand field observations at the Arda-Tek barramundi farm in Northern Australia, proved the tropical *Prymnesium parvum* strain, isolated from these ponds (PPDW02), to be highly toxic towards the gill cell line RTgill-W1. Similar to European *P. parvum* strains (Hagström and Granéli, 2005; Johansson and Granéli, 1999), our tropical strain proved most toxic during the stationary phase when exposed to nutrient limitation. While we here cannot entirely exclude the contribution of other environmental factors to the observed increase in toxicity in the nutrient deficient treatments (Baker et al., 2007; Manning and La Claire, 2010) and did not conduct C:N:P analysis, we have shown that lysed cells temporarily released large amounts of toxins that rapidly degraded even in the dark (Fig. 2.1, compare 4 and 24 h toxicity). The increased toxicity of live algal cultures 3 days after clay application in both treated and control cultures is likely due to the release of ichthyotoxins during culture senescence (Fig. 2.1, compare 4 h and 3 day cell counts), highlighting the requirement for complete removal of algal cells during the initial flocculation event. Previous investigations on clay flocculation reported *P. parvum* cell removal to be dependent upon the nutrient condition of cells, hence attributing removal of toxicity directly to the removal of algal cells and their intracellular toxins (toxicity measured as hemolytic activity Hagström and Granéli, 2005; Sengco et al., 2005). However, employing the sensitive gill cell line assay in the present work, we found no evidence of such a correlation, with identical decreases in cell density, but not toxicity observed across all nutrient conditions. Rather than removing *P. parvum* toxicity by flocculating cells, Phoslock™ (B3) clay appeared to actively adsorb water soluble *P. parvum* ichthyotoxins, thus presenting a much greater benefit than by just achieving cell or phosphorus removal. While Sengco et al. (Sengco et al., 2005) and Hagström and Granéli (Hagström and Granéli, 2005) reported increases in the hemolytic activity of *P. parvum* cultures in response to clay treatment, presumably a stress response of live cells to flocculation, we did not observe such an increase during our nutrient manipulation experiments with diluted cultures ($1-1.5 \times 10^5$ cells

mL⁻¹, pH 7.7-8.3). However, we did observe significantly increased toxicity in surface samples when high density (10⁶ cells mL⁻¹), high pH (9.8) *Prymnesium* cultures were treated with Phoslock™ clay, despite complete cell removal at the surface (Fig. 2.2). This indicates the release of intracellular toxins. Physical contact between clay particles and algal cells has previously been demonstrated to cause lysis in a range of harmful algal species, ranging from fragile raphidophytes to armoured dinoflagellates (Lee et al., 2013b; Rivera, 2014, October; Sengco, 2001; Sengco et al., 2001). Observed higher particle counts in the lower size range (<4 µm) which neither corresponded to *P. parvum* cells nor clay particles, support this suggestion, as these smaller sized particles likely represent cell fragments and/or aggregates of cell fragments and clay particles. The reduced toxin adsorption by Phoslock™ clay at higher pH explains its failure to remove intracellular toxins released through algal cell lysis and therefore the observed increase in toxicity after clay application. By comparison, the pH of the Australian barramundi ponds, to which Phoslock™ (B3) was successfully applied, was adjusted to below 7.7 (Body, 2011).

2.4.2 Clay Screening

Flocculation of *P. parvum* cells presents an important benefit of clay application, since continuous ichthyotoxin production of cells remaining after treatment would require subsequent clay additions. Previous clay screening for flocculation purposes of *P. parvum* cells by Sengco et al. (Sengco et al., 2005) showed that bentonite type clays were best suited for *P. parvum* cell removal, albeit with a removal efficiency of only 17.5% by natural clays at cell concentrations comparable to our present work (10⁵ cells mL⁻¹). While the authors were able to achieve complete removal through the addition of polyaluminium chloride (Hagström and Granéli, 2005; Sengco et al., 2005), nearly all representatives of the four clay groups assayed in the present work were able to achieve high removal efficiencies (≥80%) without the addition of chemical flocculants. Critically, cell removal proved to be a poor predictor of ichthyotoxin removal for which significant differences between clay groups at pH 7 and 9 were observed (Fig. 2.6).

The practical implications of the pH dependence of ichthyotoxin adsorption are clearly reflected in the comparative minimum effective dosages of Phoslock™ (B3)

and Ed (B5) type clay required to completely remove ichthyotoxic effects of lysed *P. parvum* preparations at the ecologically relevant pH of 9.2 (Fig. 2.7). Previously discussed observations of continuous ichthyotoxin production and release of intracellular toxins through clay particle collision mediated cell lysis highlight the importance of screening clays not just for cell flocculation, but also their ichthyotoxin adsorptive properties.

The toxin adsorptive characteristics of clay have previously only been described for bentonite type clays (Pierce et al., 2004; Prochazka et al., 2013; Seger et al., 2014; Sengco et al., 2005), employed because of their high cell removal rather than ichthyotoxin adsorptive capabilities. Our detailed screening of different clay groups revealed that in case of *P. parvum* ichthyotoxins, these toxin adsorptive properties are not only restricted to bentonite, but also encompass to a certain extent zeolite and kaolin type clays at a pH of 7. The weaker adsorption capacity of kaolin compared to bentonite has been attributed to the comparatively higher cation exchange and swelling capacity of bentonites, which not only allows molecules to be incorporated into the interlayer spaces, but also greatly increases the surface area available for absorption (Figueroa et al., 2004; Polati et al., 2006). While zeolites, similarly to bentonites, exhibit a high cation exchange capacity and possess a large surface area due to their porous structure, they are non-swelling. Interestingly, the low swelling bentonite preparation (B1), also failed to remove any ichthyotoxins. However, when considering the large variation in cell flocculation efficiencies reported in the literature between major clay types (Sengco, 2001; Sengco et al., 2001; Yu et al., 2004), it becomes increasingly clear that the performance of individual clays does not reflect general characteristics of the clay group. A large proportion of this observed variation may be due to the different levels of impurities present in available commercial clays employed in most studies (e.g. Table 2.1). Based on the lower ichthyotoxin removal at pH 9 by all clay minerals tested here (except B4-6), it appears likely that pH dependent changes in the protonation status of toxin molecules and/or the pH dependent surface charge of clay minerals is driving their adsorption kinetics (Figueroa et al., 2004; Fukahori et al., 2011; Polati et al., 2006; Stückenschneider et al., 2013). Unfortunately, due to

the poorly understood chemical nature of *P. parvum* ichthyotoxins, the contribution of the ionization state of toxin molecules cannot yet be fully resolved.

Valuable insight into the pH dependence of the adsorption process can be gained through interpretation of zeta potential measurements, an approximation of surface charge that governs the behaviour of clay particles in suspension (Chorom and Rengasamy, 1995; Moayedi et al., 2011; Saka and Güler, 2006). Clay particles are inherently negatively charged due to isomorphic substitutions in their crystal lattice and exhibit pH dependent charges on their edges (Bilgiç, 2005; Niriella and Carnahan, 2006). Deprotonation of edge sites with increasing pH gives rise to a more negative zeta potential of clays in water (Yukselen and Kaya, 2003). However, the presence of multiple charged cations of alkali earth and/or heavy metals can result in the opposite trend (in pH ranging from 7 to 10), if such cations are present either in the dispersant or on the clay surface (Chorom and Rengasamy, 1995; Saka and Güler, 2006; Yukselen and Kaya, 2003). Reason for this behavior is increased particle aggregation due to the surface coating of clay particles with insoluble carbonates at higher pH. Therefore, the drop in the absolute zeta potential value between pH 7 and 9 was expected for the majority of clays, and provided by presence of CaCl_2 in the L-15/ex medium (Schirmer et al., 1997). The zeta potential of sodium rich clays has been shown to decrease with pH (7 to 10) due to higher dispersal of individual clay particles (Chorom and Rengasamy, 1995). Accordingly, the zeta potential of the Na-bentonite B6 was observed to be greater at pH 9 than at pH 7. Another Na-bentonite, Phoslock™ (B3), has been modified with La^{3+} by the manufacturer, a trivalent cation that is known to form insoluble compounds under alkaline conditions (Ross et al., 2008). This accounts for the large observed difference in zeta potential between pH 7 and 9 (+8 mV) in this special case.

The three bentonites B4-B6 were the only clays to consistently achieve high ichthyotoxin removal at both pH 7 and 9. Interestingly, the calcium-bentonite B2, which originates from the same clay deposit as B4 and B5, failed to efficiently reduce toxicity and exhibited a much reduced surface charge at pH 9. The only difference between these clays is the depth from which they were mined. B4 and B5 originated from the lower layers of the deposit, whereas B2 was mined from the

surface and subject to weathering processes that appear to have significantly reduced its capacity to adsorb *P. parvum* ichthyotoxins.

Similarly to B2, all other clays, including Phoslock™ (B3), which exhibited lower zeta potentials at pH 9, displayed lower toxin adsorption (B3) or even exacerbated ichthyotoxicity (kaolins, zeolites and Korean loess) at pH 9, despite exhibiting zeta potentials comparable to the high adsorbing bentonites (B4-6). While we observed no direct correlation between the extent of zeta potential and ichthyotoxin adsorption, changes in clay charge at pH 7 and 9 appear to be consistent with changes in *P. parvum* ichthyotoxin adsorption.

2.5 Conclusions

Our detailed screening of fourteen different individual clays, representing four major clay mineral groups, demonstrates that the potential of clays as mitigation agents of fish-killing *Prymnesium parvum* blooms in aquaculture ponds extends beyond their phosphorus removal (valid for Phoslock™) and cell flocculating properties. Ichthyotoxin adsorption offers an important additional benefit to clay application through the removal of extracellular, as well as intracellular ichthyotoxins. This presents an important addition to the global effort to mitigate ichthyotoxic effects of *P. parvum* not only in Australia, but also abroad.

Unweathered, unmodified bentonites proved to be better suited for ichthyotoxin adsorption purposes than Phoslock™ at higher pH (9–9.8), since lower concentrations were required for complete removal of ichthyotoxicity. The removal of *P. parvum* cells remains imperative to eliminate the source of continued ichthyotoxin production, but care should be taken to select clays not solely based on their flocculating, but also their ichthyotoxin adsorptive properties to efficiently combat extracellular as well as intracellular ichthyotoxins released during the initial flocculation event through physical damage by clay particles. While reduction of aquaculture pond pH through the stimulation of microbial growth by addition of molasses could greatly increase the ichthyotoxin adsorption efficiency of Phoslock™ clay, unmodified bentonites represent a more cost effective alternative to the specialized lanthanum-modified Phoslock™ clay. Due to their world-wide

application in a range of commercial applications, unmodified, unweathered bentonite type clays are easily accessible to local fish farmers and represent a great potential on-farm emergency response tool.

Cell flocculation proved to be a poor indicator of ichthyotoxin adsorption, the extent of which cannot be explained by the major clay mineral group present, but rather through understanding of the physical and chemical characteristics of individual clays. Zeta potential measurements proved to be a good first step towards a more detailed understanding of ichthyotoxin adsorptive properties of clay minerals, revealing that pH induced changes in clay zeta potential can be indicative of changes in *P. parvum* ichthyotoxin adsorption. More detailed knowledge of the chemical composition of clay minerals and well characterized ichthyotoxins (e.g. karlotoxins, brevetoxins) will potentially provide valuable insight into the adsorption kinetics of different clay types to facilitate an efficient selection of local clays for cost-effective mitigation purposes in the future.

Chapter 3 *

Assessment of the efficacy of clay flocculation in Korean fish farm waters: *Cochlodinium* cell removal and mitigation of ichthyotoxicity

Abstract

The ichthyotoxic dinoflagellate *Cochlodinium polykrikoides* poses a significant threat to the Korean finfish aquaculture industry, thus calling for effective mitigation strategies. We here report for the first time on investigations to assess the efficacy of routine application of clay to a naturally occurring *Cochlodinium polykrikoides* bloom off Namhae Island in South Korea during August-September 2015.

Applications of conventional Korean loess reduced *Cochlodinium* cell concentrations from a maximum of 4,600 mL⁻¹ to levels considered safe for aquaculture operations (<1000 cells mL⁻¹). However, at the same time, two clay application episodes increased ichthyotoxicity in water samples (measured by the gill cell line RTgill-W1 assay) by up to 32% compared to non-treated areas. Simulated laboratory culture experiments identified clay mediated *Cochlodinium* cell lysis as the probable cause. Lysed cells maintained cytotoxicity for up to 48 h (gill cell viability reduced to 39±7% after 2.5 h exposure), but application of a finely-milled Korean loess completely eliminated ichthyotoxicity. Our results suggest that the fish-killing mechanism of *Cochlodinium* is comprised of both a stable (up to 48 h) and a transient fraction (decay within minutes). The latter indicates potential involvement of reactive oxygen species (ROS). We offer recommendations to fine-tune existing clay application regimes in Korean waters by focusing not on *Cochlodinium* cell removal, but on the adsorptive properties of clays to eliminate ichthyotoxicity.

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3.1 Introduction

Harmful algal blooms pose a significant threat to an ever expanding finfish aquaculture industry attempting to meet increased global consumer demand. The fish-killing dinoflagellate *Cochlodinium polykrikoides* is of particular concern, due to the production of highly potent, yet uncharacterised ichthyotoxins and its apparent geographic range extension across Asia, North America, Europe and the Middle East (Kudela and Gobler, 2012; Kudela et al., 2008). Nowhere has the economic and cultural impact of *C. polykrikoides* been as devastating as in Korea, where in 1995 almost 10% of the total annual finfish production equivalent to \$US100M was lost (Kim et al., 2010; Lee et al., 2013a; Park et al., 2013). Since then, annually recurrent blooms of this species have increased in frequency and continue to threaten extensive fish farm operations in the region. Starting in 1996, the Korean government implemented Korean loess dispersal (active ingredients are montmorillonite and kaolinite clay minerals; Imai et al., 2006; Kim et al., 2010; Lee, 2008; Park et al., 2013) to attempt to reduce economic damages.

First pioneered by Shirota (1989) in Japan and Yu et al. (1994b) in China, clay dispersal aims to promote the generation of rapidly sinking clay-algal aggregates that entrain additional cells during their descent. Both laboratory and field trials investigating the efficacy of this approach have reported excellent cell removal efficiencies (up to 100%), dependent upon clay type and target species, algal concentration, turbulence and clay loading (Archambault et al., 2003; Archambault et al., 2004; Beaulieu et al., 2005; Hagström and Granéli, 2005; Padilla et al., 2010; Sengco and Anderson, 2004; Sengco et al., 2001; Sun and Choi, 2004; Yu et al., 1994a).

Clay flocculation traditionally has targeted the removal of HAB cells through flocculation, but recent work has highlighted the effective adsorption of dissolved, extracellular phycotoxins to clay particles (Pierce et al., 2004; Prochazka et al., 2013; Seger et al., 2015b; Sengco et al., 2005). This property of clay deserves more attention, since physical contact between clay particles and algal cells during flocculation can cause algal cell lysis and concomitant release of intracellular

ichthyotoxins (Archambault et al., 2003; Lee et al., 2013b; Rivera, 2014, October; Seger et al., 2015b; Sengco, 2001), which can amplify fish-killing effects (Deeds et al., 2002; Dorantes-Aranda et al., 2015; Dorantes-Aranda et al., 2011). To capitalise on this property of clay requires critical knowledge of the fish killing mechanism of different harmful algal species. Most fish-killing HABs, such as *Cochlodinium*, comprise fragile cells that upon cell lysis release reactive oxygen species (ROS) that react with free fatty acids to produce highly ichthyotoxic lipid peroxidation products (Dorantes-Aranda et al., 2015; Kim et al., 1999; Marshall et al., 2003).

Working with Korean scientists during the 2015 *Cochlodinium* bloom season in the Korean South-East Sea, we here report for the first time in detail on the routine application of clay to a naturally occurring *Cochlodinium polykrikoides* bloom to assess implications for ichthyotoxicity. Cytotoxicity of field samples was newly assessed with the RTgill-W1 gill cell line assay and field observations interpreted through a series of complementary laboratory culture experiments simulating field conditions.

3.2 Methods

3.2.1 Field application of clay

3.2.1.1 Sampling location

The Namhae Island fish farm area is located in South Gyeongsang Province, South Korea (34.95° N 127.92° E, Fig. 3.1) and focuses on the cultivation of red sea bream (*Pagrus major*) in deep water (10 m) pens. In early August 2015, this fish farm was threatened by a *C. polykrikoides* bloom moving towards the farm in a South-Easterly current. The Korean Government commenced clay spraying operations on the 3rd of August and continued until 20th of September, covering an approximate area of 1 km² upstream of the farm. Locally sourced Korean loess (from Namhae Island) was continually dispersed in a rectangular pattern from a boat fitted with an internal clay dispenser (on-board mixing of clay with seawater) and additionally hosed off the back of a barge with seawater (Fig. 3.2a and b, respectively). Several smaller boats crisscrossed the wake of the clay plume to induce turbulence and thereby facilitate dispersal.

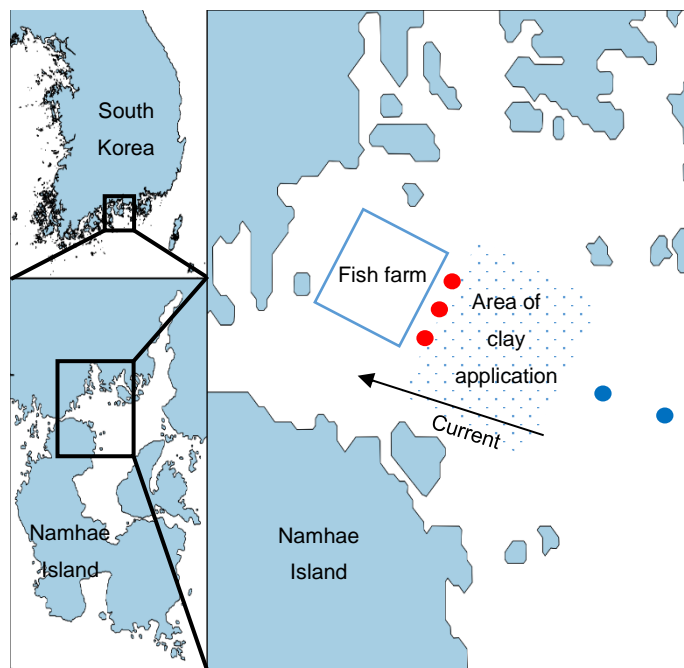


Figure 3.1 Location of the Namhae Island fish farm (blue square) and zone of clay application (dotted area). Locations of clay treated (red circles), control sampling sites (blue circles) and direction of current (arrow) are indicated.

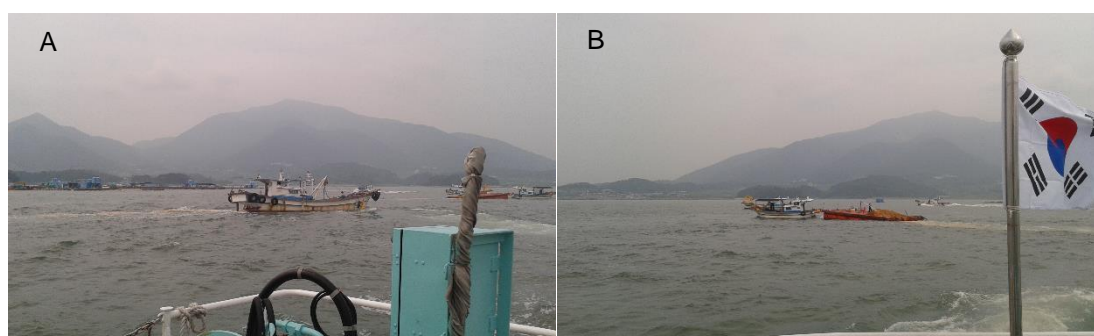


Figure 3.2 Routine clay spraying operations in the field. Korean loess dispersed via inboard clay dispenser (A) or hosed off a barge (B).

3.2.1.2 Field sampling

On day 27 and 33 during the ongoing clay application, 1 L water samples were collected using a Van Dorn water sampler at 1 m depth intervals (0.5-9 m) within the clay treated area during continuous dispersal (~ 50 m upstream of fish farm; 3 replicates) and in the untreated area (~ 1.5 km upstream; 2 replicates; see Fig. 3.1 for sampling locations). Control samples were collected 30-60 min after sampling in the clay treated area was completed. Samples were stored overnight at 25 °C in the

dark, pooled across replicates for each site (treated and control) and the next morning tested on the RTgill-W1 cell line following the protocols of Dorantes-Aranda et al. (2011) (see 2.4). Seawater collected from a depth of 9 m in the clay treated area served as the non-toxic control (containing 51 ± 41 *Cochlodinium* cells mL⁻¹). Dinoflagellate cell concentration was determined through microscopic cell counts in 0.1-1 mL of Lugol's (2%) preserved sample the following day (Nikon Eclipse TS 100; Sedgewick-Rafter chamber). Depth profiles of temperature and salinity were collected at both sites (Seabird 19plus, USA).

3.2.2 Ichthyotoxic principle

3.2.2.1 Laboratory cultures

To interpret field observations, *Cochlodinium polykrikoides* strain CP-YD-1409, isolated from Yeongdeok-gun, Korea on 16/09/2014 was grown in GSe medium prepared from filtered (0.22 µm) coastal seawater. Salinity was adjusted to 32 with deionised water and cultures maintained in 150 mL glass Erlenmeyer flasks at 20 ± 1 °C. Light was supplied at $120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a light:dark cycle of 12:12 h. The culture was harvested during the late exponential/early stationary phase and subjected to four different treatments to compare cytotoxicity of whole and lysed cells. Lysates were prepared through sonication of 10 mL of culture for 1.5 min on ice at an amplitude of 7 µm peak to peak (Probe-type sonicator; Measuring & Scientific Equipment Ltd., London, England). Samples were either immediately tested on the gill cells or stored overnight to simulate storage conditions of field samples. In another treatment, intact algal culture was gently gravity filtered through a 10 µm mesh, passed through a 0.22 µm syringe filter (PES membrane) and stored in the light for two days. All preparations (intact cells, fresh lysate, stored lysate and stored filtrate) were diluted with GSe medium to achieve final concentrations equivalent to 0-14,000 *Cochlodinium* cells mL⁻¹ before gill cell cytotoxicity assays (see 2.4). Superoxide anion production in the fresh lysate was determined within 5 min of sonication with a plate reader following the method developed by Godrant et al. (2009).

3.2.2.2 Bloom termination

During the bloom termination phase at the Namhae Island fish farm, surface samples were collected in the untreated upstream area on the 10th of September 2015 and stored in the laboratory overnight. Samples were diluted with 0.22 µm filtered coastal seawater the following morning to yield concentrations of 0-700 cells mL⁻¹ and tested on the gill cell line (see 2.4).

3.2.3 Effect of clay particle size

3.2.3.1 Particle size analysis

To determine the effect of Korean loess particle size on flocculation efficiency and cytotoxicity, conventional Korean loess (source identical to that used in field) and a finely milled Korean loess product (Haenam, Jeongwon Clay Company) were obtained and their particle size distributions determined. Loess samples of 10 g were wet sieved with deionised water through a 62 µm mesh and the <62 µm fraction suspended in 2.55 g L⁻¹ Calgon to separate the sample further into the 0-2 and 2-62 µm fractions in a 1 L sedimentation column according to Stoke's settling velocities (Folk, 1974). The resulting 2-62 µm sample was further analysed with a Coulter particle counter (Beckman, Multisizer 4) to determine particle size distribution within this fraction. The finer (<2 µm) and larger particle (>62 µm) fractions were quantified by evaporating 50 mL of clay sample to dryness (60 °C for 48 h). Final weights of these fractions were corrected for the presence of Calgon dispersant by subtracting the weight of 50 mL of oven dried Calgon solution from dried clay samples."

3.2.3.2 Ichthyotoxin adsorption

Surface Korean bloom waters collected in the control area during the first field sampling interval (~1100 *Cochlodinium* cells mL⁻¹) were partitioned among 35 mL glass test-tubes. Conventional and milled loess were suspended in deionised water, such that the addition of 2 mL to 30 mL of sample yielded final concentrations of 0.1-0.5 g L⁻¹ (3 replicates per concentration). Controls received 2 mL of deionised water. Samples were inverted once after clay addition to simulate well mixed surface waters and left undisturbed for 3 h, when 25 mL of the supernatant was

collected. Aliquots of 2 mL from each replicate were pooled (homogenised across sites) and tested on the gill cells in triplicate wells (to estimate assay error), while the remainder was preserved in 2% Lugol's for later cell counts.

3.2.3.3 Factors influencing flocculation

To achieve the *C. polykrikoides* cell biomass necessary to assess the effect of turbulence, dinoflagellate concentration and flocculation regime on cell removal efficiency, several laboratory grown Korean culture strains were combined during the late exponential/early stationary phase to give a final concentration of 3000 cells mL⁻¹. To investigate whether removal efficiency could be increased through altering the clay application regime, single large doses of clay as well as multiple, consecutive additions of 0.25 g L⁻¹ clay equalling the identical final clay loading (0.5 or 0.75 g L⁻¹) were added to 30 mL of culture at 3000 dinoflagellate cells mL⁻¹. To simulate turbulence and potential resuspension of additional clay dispersal events in the field, test tubes were inverted after each addition and received either 2 mL of clay suspension or deionised water, such that each treatment received 3 total additions/inversions at 5 min intervals. In further experiments, algal cell concentration was adjusted to 600-3000 cells mL⁻¹ and 0.5 g L⁻¹ of either milled or conventional loess added. Control cultures received 2 mL of deionised water. *Cochlodinium* cell concentration was adjusted to 1000 cells mL⁻¹ through dilution with filtered (0.2 µm) coastal seawater and divided into 35 mL glass test tubes as described above. Turbulence was induced after addition of milled or conventional Korean loess (final concentration of 0.5 g L⁻¹) through repeated test tube inversion (5 min intervals between inversions) before the 3 h incubation period. Cell removal efficiency in all designs was determined from microscopic cell counts of Lugol's preserved supernatants as follows (Eq 1):

$$RE = \left[\left(1 - \frac{Count_{treated}}{Count_{control}} \right) * 100 \right]$$

3.2.4 Gill cell viability assay

The RTgill-W1 cell line viability assays were conducted following the protocols of Dorantes-Aranda et al. (2011). Briefly, 100 µL of 1.5 x 10⁵ gill cells mL⁻¹ suspended in

L-15 medium (Sigma L1518) were seeded into the upper compartment of a specialized semi-permeable membrane system (Corning 3381), while the lower compartment received 200 μL of supporting L-15 medium. Cells were allowed to attach for 3 days at 20 °C in the dark and the L-15 replaced with L-15/ex medium (Schirmer et al., 1997) 12 h before exposure. This medium was subsequently discarded and 100 μL of sample pipetted into each well. Plates were then incubated at 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 2.5 h, before both compartments were rinsed twice with PBS. Subsequently, 100 μL of 5% resazurin (Sigma, MKAA3887) in L-15/ex was added to the upper and 200 μL to the lower compartment. Plates were incubated in the dark for a further 2 h and gill cell viability quantified by measuring the metabolic reduction of the resazurin dye to fluorescent resorufin in a plate reader at excitation and emission wavelengths of 520 and 680 nm, respectively (Glomax, Promega). Gill cell viability is reported as percentage of the respective non-toxic control.

3.2.5 Statistical analysis

Data were tested for normality and homogeneity of variance with Kolmogorov-Smirnov and Levene's tests, respectively. Gill cell viability data collected during the field applications of clay were log transformed to meet the assumptions of a three-way, model I ANOVA investigating the interaction between sampling day, depth and site. Type III sums of squares were employed, as sample size was slightly unbalanced due to single wells within the gill assay being compromised during gill seeding, as evident by presence of air bubbles prior to exposure (only 3/4 replicates for some observations). Flocculation data were analysed with two-way, model I ANOVAs. Significant effects were followed up with Tukey's HSD or Welch's two-tailed t-test. The concentration effect of field samples on gill cell viability was modelled through linear regression. P-value levels follow convention and are as follows: n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$. All values represent the mean \pm 1 standard deviation. All analysis were performed with the statistical package R (www.r-project.org).

3.3 Results

3.3.1 Field application of clay

The 2015 *Cochlodinium polykrikoides* bloom in the Korean South Sea lasted for 56 days, with maximum cell concentrations of 32,000 cells mL⁻¹ recorded. A total of 80,756 tons of clay was dispersed in the region. Daily monitoring of *Cochlodinium* cell concentrations off Namhae Island conducted by the National Institute of Fisheries Science (NIFS) recorded dinoflagellate cell concentrations between 600 and 4600 cells mL⁻¹ (Fig. 3.3). Fluctuations in maximum cell concentration reflect the irregular distribution of *Cochlodinium* within the region. Isolated bloom patches of up to 700 cells mL⁻¹ persisted until the 10th of September. The water column at both the clay treated and control site was well-mixed (no stratification), with an average water temperature of 25°C and a salinity of 31.5 (data not shown).

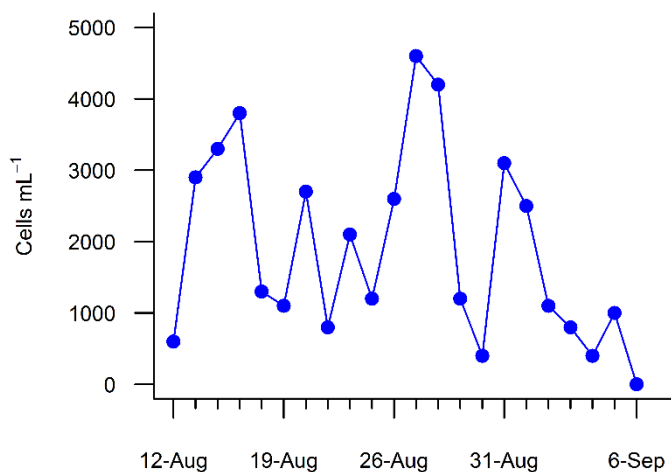


Figure 3.3 Maximum daily *Cochlodinium* cell concentrations off Namhae Island during the 2015 bloom season (data provided by NIFS).

Routine application of Korean loess at the Namhae Island fish farm significantly reduced *C. polykrikoides* cell concentrations from observed maxima of 2900 and 1740 cells mL⁻¹ (first and second sampling interval, respectively) to an average of 130±70 cells mL⁻¹ across all depths (Fig. 3.4a and c

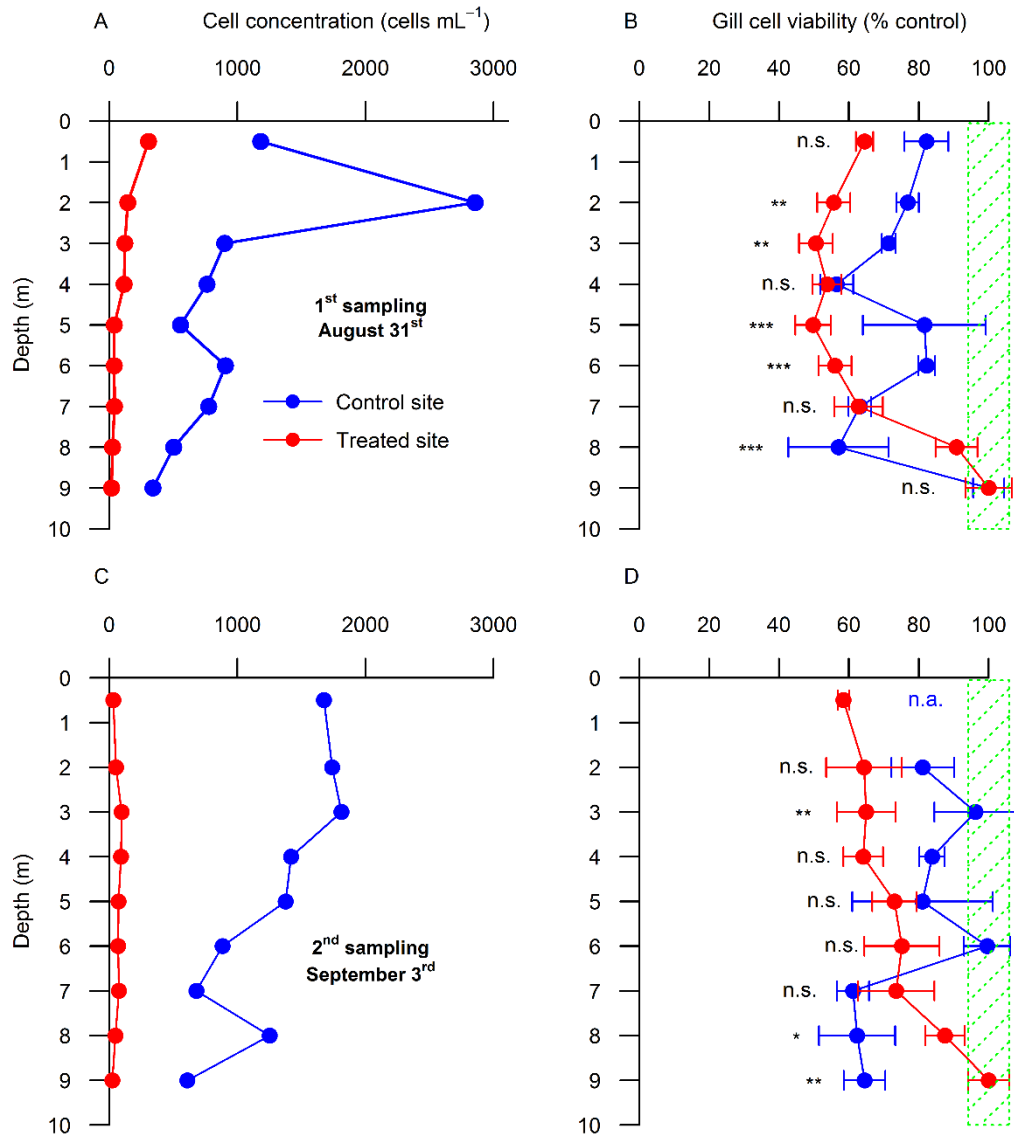


Figure 3.4 Depth profiles of *Cochlodinium* cell density and cytotoxicity towards RTgill-W1 assessed on day 27 (A, B) and 33 (C, D) of continuous clay application off Namhae Island. A and C: cell counts (cells mL⁻¹) at treated (red) and control sites (blue). B and D: toxicity of field samples towards the gill cell line RTgill-W1 (% viability of non-toxic control). Error bars represent 1 standard deviation (n=4). Asterisks denote significant differences in gill cell viability between individual depths detected with Tukey's HSD conducted on the full three-way model (n.s. = not significant; n.a = not available). Green shaded area indicates 1 standard deviation around the non-toxic control (100% viability).

). A three-way ANOVA investigating the effect of site, depth and sampling date on gill cell viability indicated toxicity towards the gill cell line RTgill-W1 to be dependent on the sampling day specific interaction of depth and site (Table 3.1). While samples collected in the upstream control area reduced gill cell viability to a minimum of $57 \pm 5\%$ (Fig. 3.4b, depth = 4 m), cytotoxicity proved depth dependent and not all samples from the control site negatively affected gill cell viability (Fig. 3.4b and d). By contrast, all samples from the clay treated area displayed consistently high cytotoxicity. This was most evident in the upper 6 m of the water column during the first sampling interval, when samples from the clay treated site reduced gill cell viability by a further 32% compared to the control site (depth = 5 m, Tukey's $p = <0.0001$). The same trend was confirmed during the second sampling period, when the observed three-way interaction was driven by sampling date specific cytotoxicity from the control site at depth (8-9 m). While no cytotoxic activity was observed at the control sites during initial sampling, significant impacts on gill cell viability were observed six days later ($64 \pm 9\%$ gill cell viability, Fig. 3.4 b and d), whereas deep samples (8-9 m) from the clay treated area proved non-toxic (as on day 1).

Table 3.1 Three-way analysis of variance (ANOVA) conducted on the main effects of site, depth, sampling day and their interaction on gill cell viability. Degrees of freedom (Df), sums of squares (type III SS) are provided.

Source	Df	SS	F	P
Site	1	0.21	14.86	<0.001
Depth	7	1.127	11.41	<0.0001
Sampling date	1	0.005	0.358	0.551
Site * depth	7	1.236	12.056	<0.0001
Site * sampling date	1	0.007	0.511	0.477
Sampling date * depth	7	0.9	9.114	<0.0001
Site * sampling date * depth	7	0.376	3.802	<0.01
Residuals	93	1.313		

3.3.2 Ichthyotoxic principle

Laboratory cultures of Korean *Cochlodinium polykrikoides* CP-YD-1409 proved highly toxic towards the gill cell line (Fig. 3.5a). Fresh culture lysate was significantly more

toxic than live algal cultures at the highest concentration tested (14,000 cells mL⁻¹), reducing gill cell viability to 10±4 and 41±4% respectively. Culture lysate exhibited a superoxide production rate equivalent to 22.15±1.75 pmol cell⁻¹ h⁻¹. Both lysate and filtrate maintained significant cytotoxicity even after storage for up to 48 h (43±4 and 35±10 % viable gill cells remaining, respectively).

Occasional isolated bloom patches of up to 700 cells mL⁻¹ persisted beyond the NIFS monitoring period during the bloom termination phase. These field samples exhibited the highest ichthyotoxicity, with as few as 140 *Cochlodinium* cells mL⁻¹ reducing gill cell viability to 80±8% (Fig. 3.5b). Only 24±3 % of gill cells remained viable after exposure to the undiluted, original field sample (700 cells mL⁻¹).

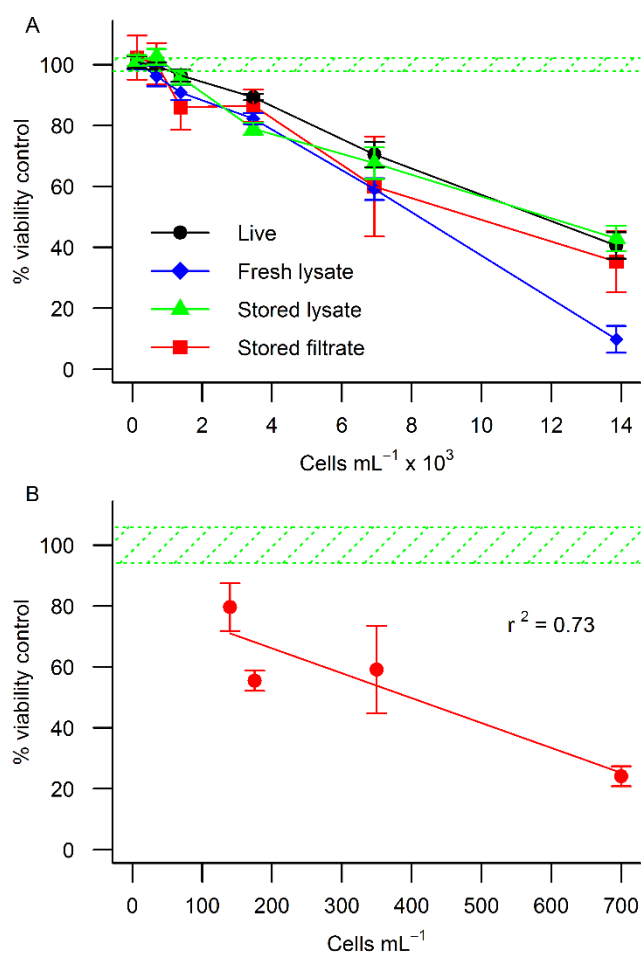


Figure 3.5 *Cochlodinium polykrikoides* cell concentration dependent ichthyotoxicity from (A) culture preparations and (B) Korean field samples collected during bloom termination. Green shaded area represents one standard deviation around the non-toxic control 9 (n=4).

3.3.3 Effect of clay particle size

3.3.3.1 Clay particle size analysis

Coulter counter analysis of the 2-62 μm size fraction revealed that in both milled and conventional Korean loess, the majority of particles was contained in the 2-10 μm range (data not shown). Complementary size separation through sedimentation indicated that finely milled loess contained nearly twice as many particles in the <2 μm range than the conventional clay product, which contained a significant amount of coarse material (41%, Table 3.2).

Table 3.2 Size distribution (% w/w) of conventional and milled Korean loess.

Size fraction (μm)	Conventional	Milled
< 2	24	44
2 - 62 μm	35	50
$> 62 \mu\text{m}$	41	5

3.3.3.2 Ichthyotoxin adsorption by clay

To interpret increased cytotoxicity in the clay treated area during field sampling, natural *C. polykrikoides* bloom waters were treated in the laboratory with both conventional Korean loess (identical to that used in the field), and the finer, milled clay product. Removal efficiency of dinoflagellate cells in 30 mL test tube incubations averaged $55 \pm 12\%$ across all treatments (Fig. 3.6a), with a two-way ANOVA indicating no significant interaction between the main effects of loess type and concentration ($F_{2,12} = 2.99$, $p = 0.089$) or their individual contributions (loess concentration: $F_{2,12} = 3.47$, $p = 0.065$ and loess type: $F_{1,12} = 1.592$, $p = 0.2311$). However, ichthyotoxicity towards the gill cells differed significantly between loess types and concentrations (Fig. 3.6b), with a two-way ANOVA indicating significant influence of both main effects and their interaction on gill cell viability (Table 3.3). Compared to the untreated, toxic control ($79 \pm 0.2\%$ gill viability, Fig. 3.6b), conventional loess exhibited 18% higher cytotoxicity than the control at a clay loading of 0.25 g L^{-1} . Even higher concentrations (0.5 g L^{-1}) did not improve gill viability (Tukey's $p = 0.26$). In contrast, application of finely milled loess at this same clay loading completely eliminated *Cochlodinium* ichthyotoxicity. Controls consisting

of seawater treated with either milled or conventional loess produced no significant effect on gill cell viability across all clay loadings (ANOVA clay type: $F_{1,29} = 0.436$, $p = 0.514$ and clay loading: $F_{1,29} = 0.436$, $p = 0.736$). Microscopic examination of *C. polykrikoides* cells directly and 3 h after loess treatment showed evidence of algal cell deformation and cell lysis (Fig. 3.7). Such cell damage was observed immediately after application of both types of loess, but proved more pronounced after treatment with conventional Korean loess employed in field applications.

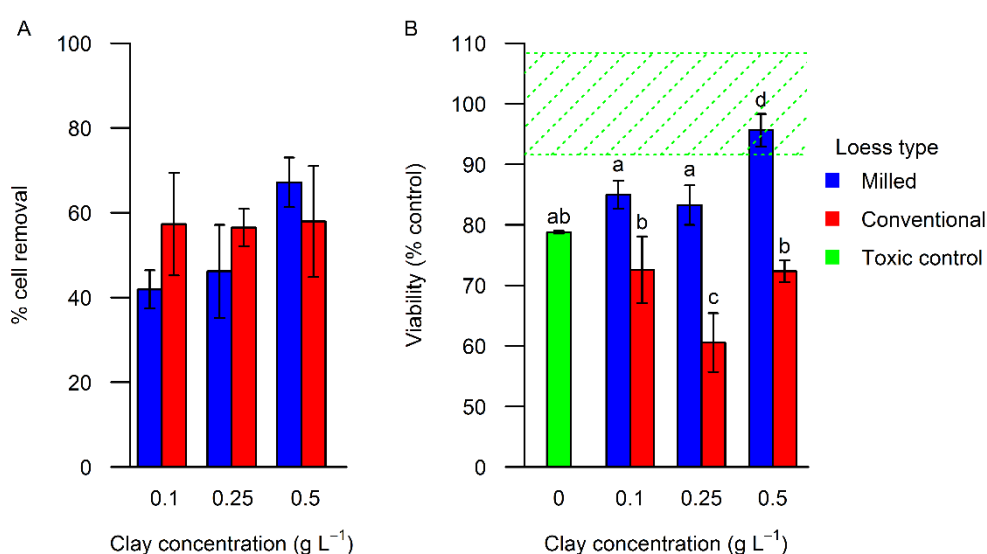


Figure 3.6 Cell removal (A) and cytotoxicity towards the gill cell line RTgill-W1 (B) by *C. polykrikoides* bloom water treated with conventional and milled loess (blue and red bars, respectively). Toxic controls received 2 mL of deionised water (green bar). Green shaded area represents 1 standard deviation around the non-toxic control (sea water + clay), with letters indicating significant differences between treatments (Tukey's HSD).

Table 3.3 Two-way analysis of variance (ANOVA) conducted on the main effects of loess type, loess concentration and their interaction on gill cell viability. Degrees of freedom (Df), sum of squares (SS) and mean square values (MS) are provided.

Source	Df	SS	MS	F	P
Loess type	1	1280.7	1280.7	127.34	<0.0001
Concentration	3	442	147.3	14.65	<0.0001
Loess type * concentration	3	539.7	179.9	17.89	<0.0001
Residuals	16	160.9	10.1		

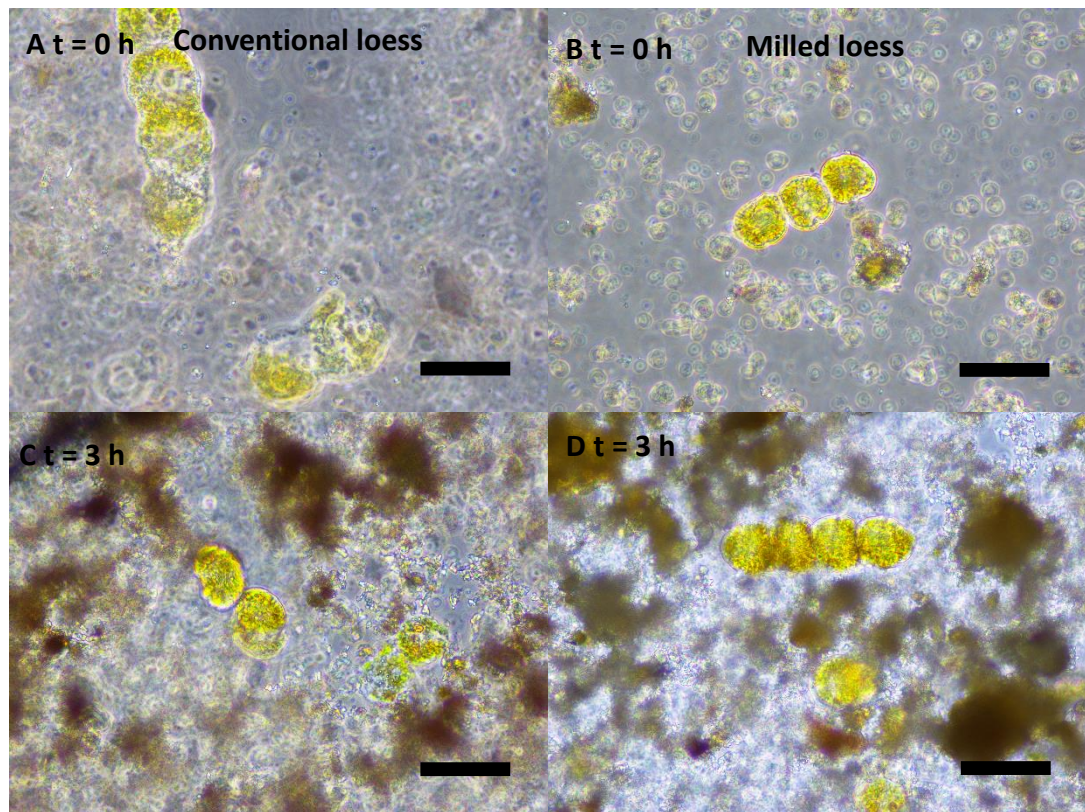


Figure 3.7 Light microscopy images (phase contrast) of *C. polykrikoides* cells entrained in clay matrix of conventional and milled Korean loess, immediately after application (A and B, respectively) and 3 h after treatment (C and D). Scale bar = 50 μm .

3.3.3.3 Factors influencing clay flocculation

We further explored the effect of turbulence, dinoflagellate cell concentration and single vs. multiple clay application on cell removal. Inducing turbulence through test tube inversion did not significantly affect cell removal efficiency (Fig. 3.8a), with a two-way ANOVA indicating no significant interaction between loess type and number of turbulence events ($F_{2,12} = 2.33$, $p = 0.140$), or turbulence itself ($F_{2,12} = 0.014$, $p = 0.989$). By contrast, cell removal was strongly dependent on algal cell density, with a two-way ANOVA proving cell removal efficiency to be significantly influenced by both the main effects of cell concentration and loess type, as well as their interaction (Table 3.4). Finely milled loess performed better than conventional clay at dinoflagellate concentrations below $1800 \text{ cells mL}^{-1}$ (Fig. 3.8b; compare

82±2% removal efficiency by milled compared to 56±4% for conventional loess at 600 cells mL⁻¹). No difference between the two clays was observed at the higher cell concentrations, where cell removal was generally much lower (only 11±10% at 3000 cells mL⁻¹; Tukey's $p = 0.82$). Removal efficiency could not be increased by replacing single large clay dosing with multiple, consecutive additions equalling an identical total clay loading (Fig. 3.8c). Only the effect of total loess concentration proved significant, with more cells being removed at a final loess loading of 0.75 (32±11%) than at 0.50 g L⁻¹ (16±11%; Welch's t-test: $p = 0.00135$).

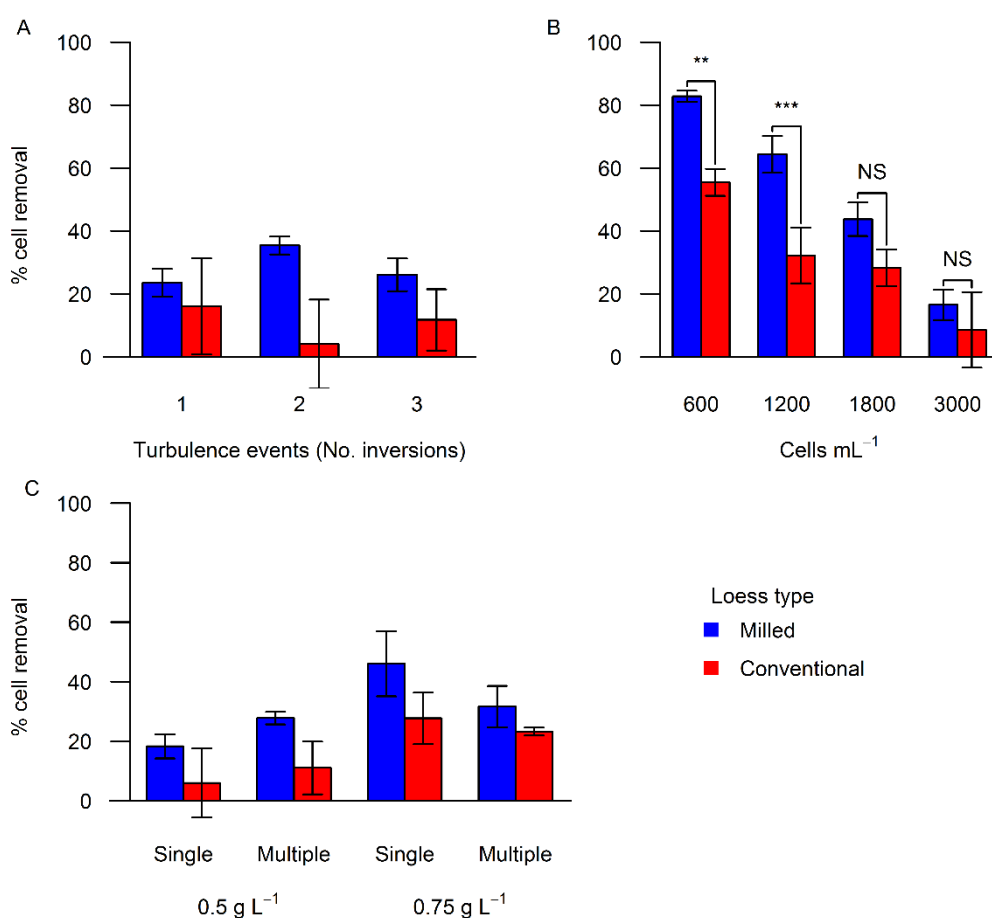


Figure 3.8 Influence of turbulence (A), cell concentration (B) and loess application regime (C) on removal efficiency of *C. polykrikoides* cells by conventional and milled Korean loess (blue and red bars, respectively). Error bars represent 1 standard deviation (n=3). Asterisks represent significant differences in removal efficiency between clay types at each cell concentration (Tukey's HSD).

Table 3.4 Two-way analysis of variance (ANOVA) conducted on the main effects of clay type, *C. polykrikoides* cell concentration and their interaction on gill cell viability. Degrees of freedom (Df), sum of squares (SS) and mean square values (MS) are provided

Source	Df	SS	MS	F	P
Clay type	1	2572	2572	56.667	<0.0001
Concentration	3	10072	3357	73.96	<0.0001
Clay type * concentration	3	548	183	4.027	0.026
Residuals	16	726	45		

3.4 Discussion

Clay application has been routinely practiced in Korea as a HAB mitigation strategy since 1996, yet no or very limited data on its efficacy is available in the open literature. Early Korean work reported high *Cochlodinium polykrikoides* cell removal in the field after yellow loess application (Choi et al., 1998; Na et al., 1996).

Application of locally sourced Korean loess during a naturally occurring *Cochlodinium polykrikoides* bloom in August-September 2015 threatening aquaculture operations off Namhae Island reduced dinoflagellate abundance to levels below those considered dangerous to finfish aquaculture (<1000 cells mL⁻¹; NIFS). A total of 80,756 tons of clay was dispersed which is claimed to have limited overall fisheries damages to \$US4.6M. The efficacy of Korean clay application in terms of reducing ichthyotoxicity had not previously been assessed, however.

Using the RTgill-W1 cell line viability assay on natural *Cochlodinium polykrikoides* water samples during two separate clay spraying exercises, we newly demonstrated that indiscriminate clay application can inadvertently increase ichthyotoxicity of water samples, with clay treated water being up to 32% more toxic than water from control sites. This was partially confirmed by the continuing loss of 1.4M fish at the Namhae Island farm (equivalent to \$US1.2M) during the clay dispersal period. Simulated laboratory experiments with natural bloom waters pointed to clay mediated algal cell lysis as the probable cause. Microscopic examination of *C. polykrikoides* cells immediately after clay treatment confirmed the incidence of

numerous lysed cells, with freshly lysed preparations of laboratory cultures (Fig. 3.6) proving significantly more toxic than intact cells. Cell lysis in response to physical contact with clay particles has previously been reported for a variety of harmful algal species, such as *Chattonella marina*, *Alexandrium catenella*, *Prymnesium parvum* and notably *Cochlodinium polykrikoides* (Lee et al., 2013b; Mooney et al., 2011; Rivera, 2014, October; Seger et al., 2015b; Sengco, 2001; Sengco et al., 2001; Shirota, 1989). Cell lysis has been repeatedly demonstrated to amplify fish-killing effects in a variety of harmful algal species (Deeds et al., 2002; Dorantes-Aranda et al., 2015; Dorantes-Aranda et al., 2011; Marshall et al., 2003) and can be exacerbated by co-application of clay with algicides, such as sophorolipid (Sun et al., 2004a), copper sulphate and electrolytically generated sodium hypochlorite (Ebenezer et al., 2014; Jeong et al., 2002).

Despite its impacts on aquaculture operations, the exact toxic principle of *C. polykrikoides* has remained elusive. Our results with Korean *Cochlodinium* strain CP-YD-1409 suggest that its cytotoxic principle is comprised predominantly of a stable, but also a minor, short-lived fraction. Significant toxicity towards the gill cell line persisted in filtered and lysed preparations for up to 48 h, although these preparations proved slightly less potent than fresh lysate (~30% difference in gill viability; Fig. 3.3). Interestingly, this slight reduction in cytotoxicity matched the difference in viability of gill cells exposed to fresh lysate and live cells.

Ichthyotoxicity by North American *C. polykrikoides* isolates was claimed to be transient (decay within minutes), presumably due to the involvement of short-lived reactive oxygen species (ROS; Tang and Gobler, 2009). Kim et al. (1999) detected significant production of H_2O_2 and O_2^{-1} in two Korean strains. In the present work we confirmed this for Korean strain CP-YD-1409, which produced levels of O_2^{-1} comparable to the well-known high ROS producer *Chattonella marina* (Dorantes-Aranda et al., 2015). While we did not measure O_2^{-1} production in the CP-YD-1409 culture lysates stored for 48 h, its activity would have been rapidly lost after cell lysis due to destruction of cellular structure and short life span of generated O_2^{-1} (Fridovich, 1983; Halliwell and Gutteridge, 2015).

Kim et al. (2002) failed to detect ROS in the two Japanese strains they examined, instead proposing that fish-killing effects are caused by a potent cocktail of biologically active metabolites and mucus substances, such as polysaccharides. As Kudela and Gobler (2012) point out, polysaccharides tend to accumulate as cultures age, whereas ROS are predominantly produced during exponential growth (Kim et al., 1999). The age of experimental cultures therefore needs to be carefully considered and may explain the presence of both a stable as well as a transient cytotoxic fraction in our culture tested at the interphase of exponential and stationary phase. Tang and Gobler (2009) found North American isolates to exhibit highest ichthyotoxicity during exponential growth, while we found Korean field samples to be most toxic during final bloom stages. These contrasting findings may partly be explained by strain-specific ecophysiological differences between the Korean and North American *Cochlodinium* (Gobler et al., 2008; Iwataki et al., 2008). Potential strain specific responses within geographic areas and between bloom years cannot be excluded (only CP-YD-1409 strain was assessed here). This highlights how algal bloom mitigation strategies need to be fine-tuned not only for differing HAB species, but also differing geographic populations. Effective approaches should be based on a detailed understanding of the fish killing mechanisms.

In our case, ichthyotoxins released through cell lysis could be completely removed by the application of not conventional, but finely-milled Korean loess of smaller clay particle size (<30 μm , Fig. 3.3). Studies investigating the adsorption of herbicides and antibiotics to clay particles in terrestrial systems attribute the increased reactivity of finer clay particles to their higher specific surface area (Petersen et al., 1996; Santamarina et al., 2002; Thiele-Bruhn et al., 2004; Tsai et al., 2003). Particle diameter also influences the hydrodynamic properties of sinking clay particles, with higher algal cell removal efficiencies being reported for the finer particle size fraction (Louzao et al., 2015; Sengco et al., 2001; Yu et al., 1994b). Adding small amounts of fine particles can dramatically alter the adsorptive behaviour of coarse soils (Santamarina et al., 2002). During our laboratory studies, milled Korean loess demonstrated increased cell removal efficiency, but the application regime did not

significantly influence cell removal. Single, large doses ($0.5\text{--}0.75\text{ g L}^{-1}$) proved just as effective as multiple, small additions amounting to an identical final clay loading. Although initial turbulence (induced in the field by boats crossing the wake of clay dispersing vessels) appears to be an important factor contributing to the success of clay application (Sengco et al., 2005), additional agitation of 30 mL samples in the laboratory did not improve cell removal and may have facilitated escape of algal cells from resuspended flocs (Archambault et al., 2003; Beaulieu et al., 2005; Sengco et al., 2001). Only *Cochlodinium* cell concentration significantly influenced overall cell removal, with higher efficiencies being achieved at lower biomass (Fig. 3.7).

Particle size has long been recognised as an important factor determining cell removal efficiency during Korean field applications and specially designed clay dispensers have been developed for shipboard grinding of clay prior to dispersal (Kim et al., 2010; Park et al., 2013). However, due to the high initial purchasing and operating costs of these large, patented units, their availability is limited and use restricted to high priority bloom areas. Smaller blooms, such as the one off Namhae Island (\$US1.2M dead fish), were only treated with conventional clay and inadvertently may have amplified ichthyotoxic effects.

Since its implementation in Korea in 1996, clay spraying has been conducted at all at-risk aquaculture sites and therefore no untreated control mortality areas are available to reliably assess treatment efficacy. While overall aquaculture losses in Korean waters reputedly have been reduced by 80%, yearly fisheries damages vary disproportionately to *C. polykrikoides* biomass (Park et al., 2013). Numerous studies have demonstrated harmful algal cell removal efficiency to be dependent upon current flow, local loess type/particle size, dispersal method and physiological cell condition (Archambault et al., 2003; Beaulieu et al., 2005; Hagström and Granéli, 2005; Park et al., 2013). Combined with environmentally modulated variations in bloom duration and ichthyotoxin production, these factors likely contributed to the year and site specific success of clay dispersal with conventional loess and highlight the potential for improved treatment efficacy through fine-tuning of existing approaches. Our work conclusively demonstrates the benefits of employing the finer, milled loess, but the much higher costs associated with clay milling (up to 60

times higher; Park et al., 2013) would render this product uneconomical for prolonged routine application (usually 30+ days). Instead, we recommend more targeted emergency treatments with the finely-milled clay product to be applied when *C. polykrikoides* poses the greatest threat to fish health. From a practical aquacultural standpoint, identifying conditions contributing to cell lysis (e.g. wind/wave induced turbulence) and bloom stage (regional cell count data is freely available from the National Institute of Fisheries Science) could serve as indicators of when to apply more costly, but highly effective finely milled clay treatments.

3.5 Conclusions

Routine field applications with conventional, locally sourced Korean loess effectively reduced *Cochlodinium polykrikoides* cell concentrations to levels considered safe for aquaculture operations, but inadvertently increased ichthyotoxicity through clay particle mediated algal cell lysis. *Cochlodinium* cultures were strong producers of short-lived reactive oxygen species, yet significant ichthyotoxicity persisted in the seawater medium for up to 48 h after cell lysis. Our results suggest that the toxic principle of the here examined Korean *Cochlodinium* strain is comprised of both a stable and a transient fraction, the nature of the latter indicating involvement of ROS. Clay particle size proved critical to the successful outcome of clay treatment, with finely-milled loess completely eliminating ichthyotoxicity. The ichthyotoxin adsorptive properties of clay should be carefully considered when applying conventional loess to avoid inadvertent amplification of fish-killing effects. However, costs associated with loess processing limit the extent to which the finely milled product can be applied in the field. Targeting critical bloom stages (e.g. senescence) when *C. polykrikoides* ichthyotoxicity is highest, will result in higher treatment efficacy. Continuing research into the fish-killing mechanism of *Cochlodinium polykrikoides* should underpin continuing fine-tuning of traditional Korean clay dispersal strategies.

Chapter 4

Removal of extracellular *Karlodinium veneficum* and *Karenia mikimotoi* ichthyotoxins by clay minerals

Abstract

Adsorption of fish-killing algal toxins by clay minerals offers promise as harmful algal bloom mitigation strategy for fish farms, but its efficiency is clay and algal species specific. We here screened several different clay types (kaolin, zeolite, Korean loess and six bentonites) for their adsorptive capacity of extracellular *Karlodinium veneficum* and *Karenia mikimotoi* ichthyotoxins. Bentonites of small particle size (<5 µm) and high swelling capacity (>20 mL/2g) proved best suited for ichthyotoxin removal (100% removal). Korean loess and zeolite were ineffective for removal of cytotoxins produced by both species. Kaolin only demonstrated effective removal in case of *K. mikimotoi*, but concentrations required for complete removal of cytotoxicity were 5 times those required for bentonite (0.1 g L⁻¹). Complete elimination of *K. veneficum* and *K. mikimotoi* toxicity towards the rainbow trout gill cell line RTgill-W1 was achieved.

4.1 Introduction

The cosmopolitan dinoflagellate species *Karlodinium veneficum* and *Karenia mikimotoi* are well known for their deleterious impacts on wild and farmed fish (Imai et al., 2006; Place et al., 2012). Early investigations into the toxic principle of *K. veneficum* revealed that these algal cells release potent, ichthyotoxic (fish-killing) substances that act on the gill membrane (Deeds et al., 2002; Mooney et al., 2010). Isolated and chemically characterised in subsequent studies (Bachvaroff et al., 2008; Peng et al., 2010; Van Wagoner et al., 2008), these substances are now collectively referred to as karlotoxins (Place et al., 2012). The mode of action and chemical identity of *K. mikimotoi* ichthyotoxin(s), on the other hand, has remained elusive (Brand et al., 2012; Rasmussen et al., 2016). Continued research efforts to elucidate its ichthyotoxic principle have proposed potential involvement of reactive oxygen

species (Yamasaki et al., 2004), free fatty acids (Chen et al., 2011; Mooney et al., 2007), gymnocins (Satake et al., 2002; Satake et al., 2005) and gymnodinime (Seki et al., 1995). Similarly to *K. veneficum* (Place et al., 2012), crude extracts of *K. mikimotoi* exhibited high lytic activity towards erythrocytes (Neely and Campbell, 2006; Zou et al., 2010).

Despite the well-established ichthyotoxic and economic impact of these two dinoflagellate species, no effective mitigation strategies currently exist for fish farms threatened by these harmful algal species. A promising approach is the application of clay minerals for ichthyotoxin adsorptive purposes, introduced previously for the haptophyte *Prymnesium parvum* (Seger et al., 2015a, Chapter 2). Clay minerals are diverse in their physicochemical properties and their interactions with organic molecules are consequently highly complex (Lagalya et al., 2013; Moyo et al., 2014). Intrinsic clay properties, such as cation exchange capacity, surface charge and density, swelling capacity, surface area, as well as charge, size, and structure of the adsorbate govern the complex adsorption of organic molecules to clay surfaces (Gao and Pedersen, 2005; Henrichs and Sugai, 1993; Jahed et al., 2014; Moyo et al., 2014; Polati et al., 2006; Shen, 2002).

Prymnesium cytotoxicity was most effectively removed through application of bentonite clay, particularly those of higher purity (Chapter 2). A readily measured indicator of bentonite clay purity often employed in industrial applications is the swell index (capacity of clay to swell upon contact with water, Ahonen et al., 2008). Water molecules readily penetrate into the space between clay layers, forcing them apart and allowing access to the interlayer space (Hensen and Smit, 2002; Liu et al., 2016). This process significantly increases the clays' reactive surface area and allows for intercalation (incorporation) of organic molecules into the hydrophobic interlayer space (Jahed et al., 2014; Lambri et al., 2010; Moyo et al., 2014; Yukselen and Kaya, 2008). Selecting for finer clays may further increase reactive surface area and enhance ichthyotoxin adsorption (Chapter 3, Seger et al., 2017).

We here screened individual representatives of four major clay groups (zeolite, kaolin, bentonite and Korean loess) for both *Karlodinium veneficum* and *Karenia mikimotoi* ichthyotoxin removal. Ichthyotoxicity was assessed with the RTgill-W1 gill

cell line assay (Dorantes-Aranda et al., 2011). To better interpret differences in ichthyotoxin adsorption between bentonite clays discovered in Chapter 2, further testing of five physicochemically distinct bentonite clays focused on adsorption of the chemically well characterised *K. veneficum* karlotoxins (KTX). Various manipulations, such as washing of clay with deionised water and reduction of clay particle size through sonication were employed to better understand and interpret ichthyotoxin adsorption to clay particles.

4.2 Methods

4.2.1 Algal cultures

Karenia mikimotoi strain KMWL01 (South Australia) and *Karlodinium veneficum* strain KVSRO1 (Swan River, Australia) were grown in 250 mL glass flasks containing 150 mL of filtered coastal seawater (0.22 μm) at a salinity of 30. The media were enriched with GSe nutrients and cultures maintained at 20°C with light supplied at 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a 12:12 h light dark cycle.

4.2.2 Clay material

Clays were sieved through a 62 μm mesh to obtain a uniform size fraction. Physical properties of kaolin (Snobrite C), zeolite (ANZ38) and Korean loess are described in the previous chapter (Chapter 2, section 2.1). Properties of commercially available bentonites B1-B6 are summarised in Table 4.1. Swell index (capacity of clay to swell upon contact with water) was measured according to standard ASTM- D5890. Briefly, 2 g of dry clay were dispersed in 100 mL of deionised water in 0.1 g increments (10 min between additions). Samples were left undisturbed for 24 h, at which time the level of the settled and swollen clay was recorded to the nearest 0.5 mL (results are given in Table 4.1). To prepare clay slurries used in ichthyotoxin adsorption experiments, clay was suspended in deionised water (milliQ) at a concentration such that the addition of 20 μL to 980 μL of lysate achieved the final concentrations given.

Table 4.1 Physical properties of bentonites (as supplied by manufacturer).

Clay	B1	B2	B3	B4	B5	B6
Product name (origin)	Mantuan (AUS)	Arumpo (AUS)	Trubond (AUS)	Ed (AUS)	Na-bent (AUS)	Wyoming (USA)
CEC (meq/100g)	90-102	N/A	82	85.8	>50	>80
% Montmorillonite	N/A**	96	74	83.4	~90	>94
Major cation	Ca ⁺⁺	Na ⁺	Na ⁺	Ca ⁺⁺	Na ⁺	Na ⁺
Swell Index*	8	7-12	7	22	20	24

*Assessed as per ASTM- D5890 standard method.

**Not available.

4.2.3 Clay screening

Karlodinium veneficum cultures were harvested during the early stationary phase at 110,000 cells mL⁻¹ and sonicated on ice for 25 min with a probe-type sonicator at an amplitude of 7 μ m peak-peak (Measuring & Scientific Equipment Ltd., London, England). Sonication, as verified through Coulter Counter counts (Beckman Multisizer Z4), resulted in a cell lysis efficiency of 90%. Lysate pH was adjusted to 8.5 with 0.1 M HCl and 980 μ L transferred to 1.5 mL Eppendorf tubes. A volume of 20 μ L of clay slurry was added to achieve final kaolin, zeolite, Korean loess and bentonite concentrations of 0-0.5 g L⁻¹. Controls consisted of GSe medium (pH 8.5) and received either 20 μ L of deionised water or clay at 0.05-0.5 g L⁻¹. Samples were vortexed on high for 5 s before centrifugation at 9300 x g for 1 min (Thermo Fisher Scientific, IEC Micromax 3591). The resulting supernatant was tested on the gill cell line RTgill-W1 in quadruplicate and exposures conducted for 3.5 h in the light (120 μ mol m⁻² s⁻¹) in semipermeable transwell insert plates (Corning 3381). The assay technique followed Dorantes-Aranda et al. (2011) and is described in detail in Chapter 2 (section 2.4). Screening of kaolin, zeolite and Korean loess for *K. mikimotoi* ichthyotoxin adsorption followed the above protocol with the following exceptions: *K. mikimotoi* culture of 50,000 cells mL⁻¹ was concentrated through centrifugation of 40 mL of culture at 1400 x g (Hettich Universal 16A) for 5 min. The top 20 mL were discarded and the remaining culture sonicated for 10 min on ice (97% lysis efficiency). Lysate pH was adjusted to 8.5 with 0.1 M NaOH and treated with clay slurries as described for *K. veneficum* above.

To test whether substances released by the clay minerals influenced gill cell viability, 0.125 g of bentonite B1-B6 was added to 10 mL of deionised water. A

volume of 3 mL of the resulting slurry was added to a 15 mL centrifuge tube, which was subsequently centrifuged at 1400 x g for 10 min (Hettich Universal 16A). The overlaying 2.5 mL were collected for later testing and a further 2.5 mL of deionised water added to resuspend settled clay particles via vortexing. This washing process was repeated five times. The initial wash (rinsate), washed clay (product of final wash) and untreated clay were then added to lysed *K. veneficum* culture (100,000 cells mL⁻¹) and the adsorption experiment carried out as described above.

4.2.4 Particle size

To determine the effect of sonication on clay particle size and volume, 1 mL of 12.5 g L⁻¹ bentonite B2-B4 suspended in deionised water was sonicated for 0, 5, 10 and 20 min at an amplitude of 7 µm peak-peak. A volume of 100 µL of the sonicated product was added to 19.9 mL of filtered seawater (0.22 µm) and the individual particle size and volume spectra analysed with a Coulter Counter equipped with a 30 µm aperture tube (Beckman, Multisizer Z4). Initial results suggested that a sonication time of 20 min achieved the most comparable volume/size spectra between the four bentonites.

To test the effect of particle size on *K. veneficum* ichthyotoxin adsorption, 15 mL of culture were harvested during the stationary phase (150,000 cells mL⁻¹) and pH adjusted to 8.5. A volume of 1470 µL of culture lysate and 30 µL of clay were added to an Eppendorf tube to give a final concentration of 0.1 g L⁻¹ of sonicated B1-B4 (20 min). Subsequent steps were as described above.

4.2.5 Statistical analysis

Data were tested for normality and homogeneity of variance with Kolmogorov-Smirnov and Levene's tests, respectively. As indicated by these tests, bentonite screening data required log- and washed bentonite data square root transformation to meet ANOVA assumptions. Significant effects were followed up with Tukey's HSD. All values represent the mean \pm 1 standard deviation. All analysis were performed with the statistical package R (www.r-project.org).

4.3 Results

4.3.1 Clay screening

Lysed dinoflagellate cell preparations of *Karlodinium veneficum* and *Karenia mikimotoi*, proved highly toxic towards the gill cell line, reducing viability down to 8 ± 2 and $62 \pm 10\%$, respectively during the 3.5 h exposure (Fig. 4.1). *Karlodinium veneficum* toxicity towards the gill cells could be completely eliminated through treatment with 0.1 g L^{-1} of bentonite, while Korean loess, zeolite and kaolin displayed no such beneficial effect in terms of improving gill cell viability (Fig. 4.1a). Similarly, application of bentonite to the less potent *K. mikimotoi* completely eliminated cytotoxicity, whereas Korean loess and zeolite again displayed no significant improvement for gill cell viability (Fig. 4.1b). In case of *K. mikimotoi* preparations, kaolin clay also proved effective in reducing cytotoxicity, although higher final clay loadings (5 times higher) were required than for bentonite.

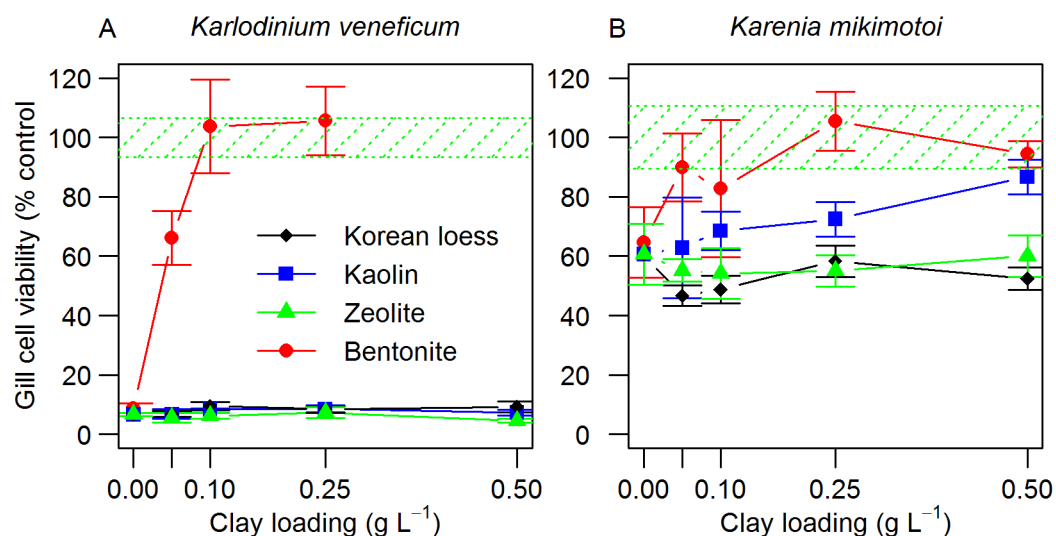


Figure 4.1 Viability of gill cells after exposure to lysed preparations of *Karlodinium veneficum* (A) and *Karenia mikimotoi* (B) treated with 0-0.5 g L^{-1} of bentonite (B4), Korean loess, zeolite and kaolin clays. The green shaded area represents 1 standard deviation around the non-toxic control (sea water + clay) and error bars 1 standard deviation around the mean (n=4).

However, further screening of several different bentonites for removal of *K. veneficum* toxicity indicated that not all bentonites could significantly improve gill cell viability (Fig. 4.2a). At a clay loading of 0.25 g L⁻¹, clays B1-B3 significantly increased gill cell viability when compared to the toxic control (Tukey's $p < 0.002$), albeit only to a maximum of 10±1% in case of B3. By comparison, bentonites B5 and B6 nearly completely eliminated toxicity towards the gill cells at this clay loading, increasing gill viability to 88±4%. Multiple, consecutive washing steps of these bentonites with deionised water had no significant effect on gill cell viability (Fig. 4.2b). As previously observed (Fig. 4.2a), B1-B3 performed poorly in terms of cytotoxicity reduction, while bentonites B4-B6 completely eliminated toxicity towards the gill cells, regardless of whether clays were untreated (natural) or rinsed with deionised water (Tukey's $p = 0.35$). The resulting rinsate also had no significant effect on gill cell viability when compared to the toxic control (Tukey's $p = > 0.06$).

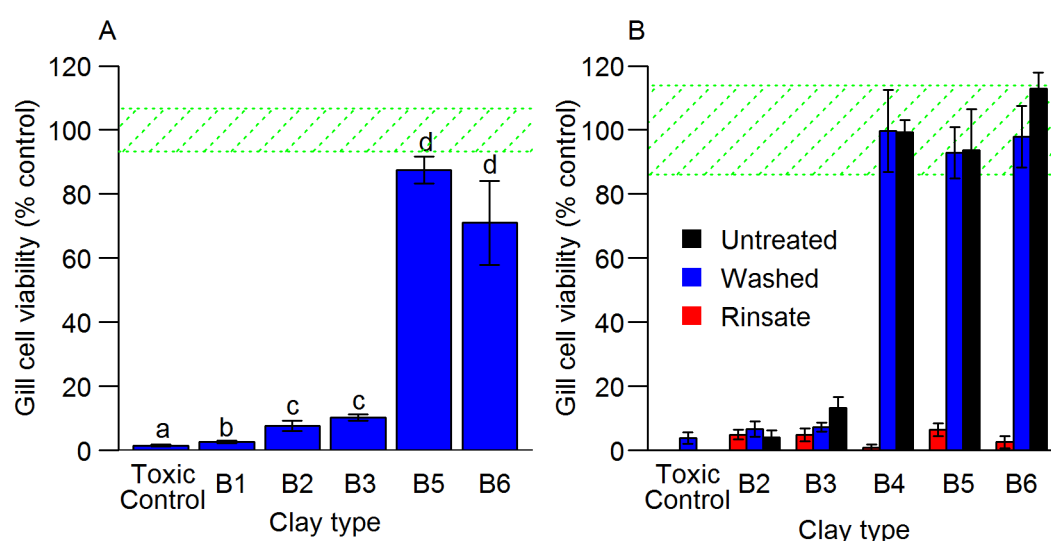


Figure 4.2 Gill cell viability after exposure to lysed *K. veneficum* preparations treated with different bentonite types (B1-B6) at 0.25 g L⁻¹; (A) natural, untreated bentonites; (B) Bentonite clays (B2-B6) washed with deionised water (blue bars), left untreated (black bars) and resulting rinsate (red bars). Error bars represent 1 standard deviation (n=4) and letters significant differences between treatments (Tukey's HSD). The green shaded area represents 1 standard deviation around the non-toxic control (sea water + clay).

4.3.2 Clay particle size

Sonication of bentonite clay suspensions significantly reduced the particle size and particle volume distribution of all bentonite clays assayed (Fig. 4.3). The extent of particle size reduction strongly depended upon sonication time (exemplified here for B2). With increasing sonication time, the particle count in the $<1\ \mu\text{m}$ fraction significantly increased (Fig. 4.3a) and the broad peak at $t=0$ in total particle volume initially dominated by larger volume particles ($>5\ \mu\text{m}$) sequentially shifted to smaller particle sizes (Fig. 4.3b).

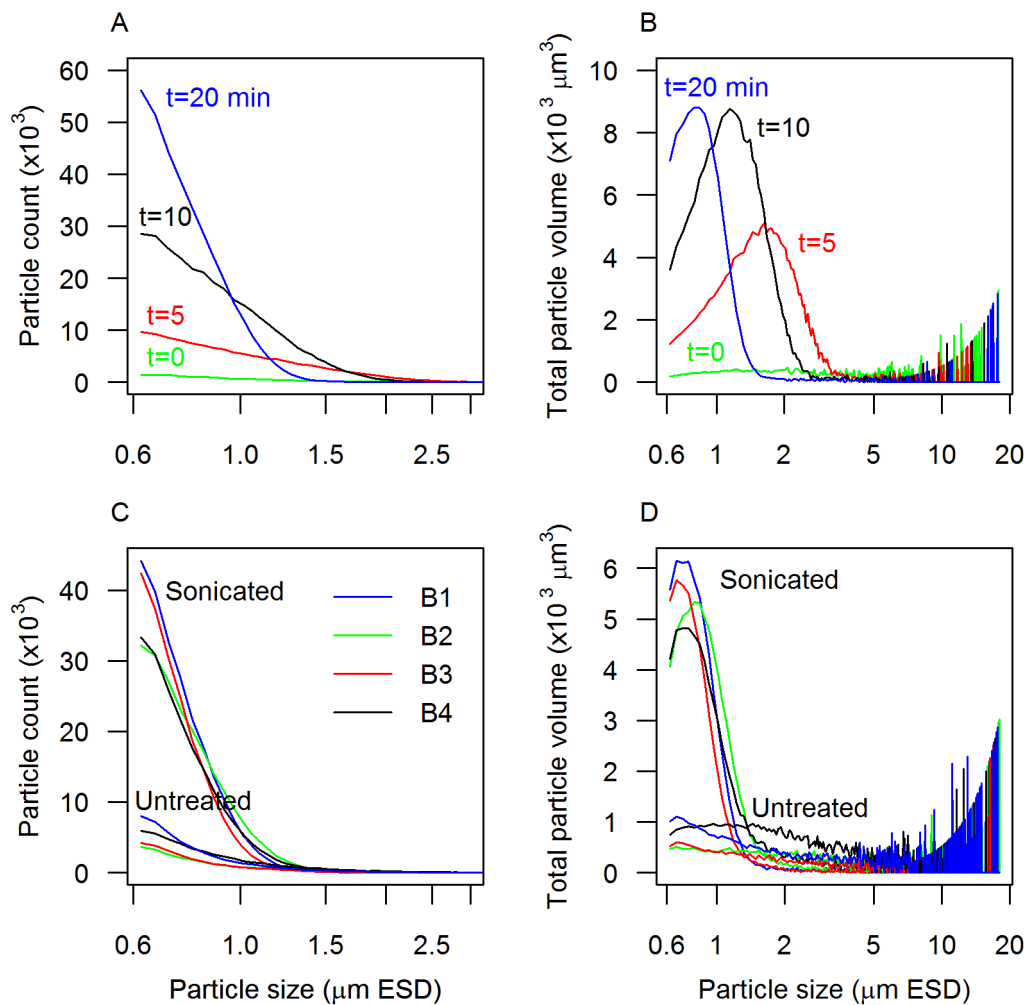


Figure 4.3. Effect of sonication time (0-20 min) on particle count (A) and total volume (B) distribution of clay type B2; (C and D) Count and volume profiles for bentonite clays B1-B4 sonicated for 0 (untreated) or 20 min (treated). Particle size is represented as μm ESD (equivalent spherical diameter).

The sonication profile of B2 proved representative of all other bentonite clay types tested (B1,B3 and B4), and comparable particle counts and volumes could be obtained for all bentonites after 20 min of sonication (Fig. 4.3c and d) to allow screening of clay particles with different size ranges for ichthyotoxin removal efficiency.

The effect of clay sonication on reduction of *K. veneficum* toxicity towards the gill cell line RTgill-W1 proved to be specific for the bentonite type (Fig. 4.4). Untreated or sonicated clay preparations of B1-B3 did not significantly influence gill cell viability when compared to the toxic control (Tukey's $p = >0.15$). However, sonication of clay type B4 significantly improved gill cell viability ($73 \pm 12\%$).

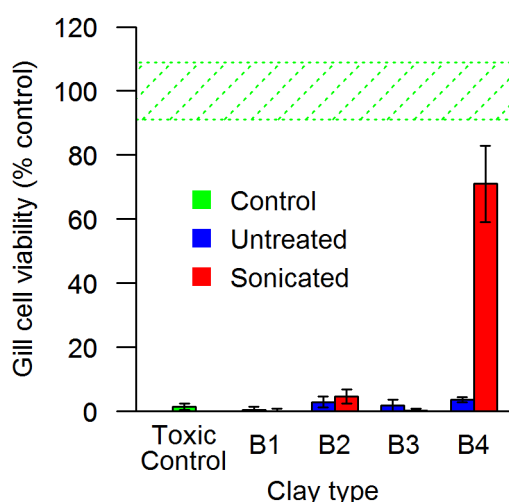


Figure 4.4. Viability of rainbow gill cells after exposure to lysed *K. veneficum* preparations treated with either sonicated (red bars) or untreated (blue bars) bentonite clays (B1-B4) at 0.05 g L^{-1} . The green shaded area represents 1 standard deviation around the non-toxic control (sea water + clay) and error bars 1 standard deviation around the mean (n=7).

4.4 Discussion

The harmful dinoflagellates *Karlodinium veneficum* and *Karenia mikimotoi* are well known for their fish-killing effects (Imai et al., 2006; Place et al., 2012), which can be greatly amplified by conditions (e.g. cell lysis) that stimulate the release of intracellular ichthyotoxins (Deeds et al., 2002; Dorantes-Aranda et al., 2015;

Mooney et al., 2010). In the present work, we conclusively demonstrated that the toxicity of thus released ichthyotoxins towards the gill cell line RTgill-W1 could be completely and effectively eliminated through targeted clay treatment.

While the gill cell damaging effect of *K. veneficum* can be attributed to the now well described karlotoxins (Bachvaroff et al., 2008; Mooney et al., 2011; Peng et al., 2010; Van Wagoner et al., 2008), the mechanisms of *K. mikimotoi* ichthyotoxicity are much less defined (Brand et al., 2012; Rasmussen et al., 2016). Various chemical compounds have been proposed (Satake et al., 2002; Satake et al., 2005; Seki et al., 1995) and significant production of both polyunsaturated fatty acids (PUFAs, 45% of total fatty acids, Mooney et al., 2007) and the reactive oxygen species (ROS) superoxide (detected only; not quantified) and H₂O₂ (1.4±0.1 nmol min⁻¹ 10⁴ cells⁻¹) reported (Yamasaki et al., 2004). Best described for *C. marina*, ROS and PUFA may act synergistically to yield a variety of highly toxic lipid peroxidation end-products (Dorantes-Aranda et al., 2013; Dorantes-Aranda et al., 2015; Marshall et al., 2003).

Improved gill cell viability in response to clay treatment lysed *K. veneficum* almost certainly is due to the direct adsorption of karlotoxins onto the clay minerals. Rinsate collected from multiple consecutive clay washes with deionised water afforded no protection of gill cells against *K. veneficum* ichthyotoxins and only direct treatment with either untreated or washed clay significantly improved gill cell viability. Adsorption experiments with purified karlotoxins conclusively demonstrated that once bound to the clay surface, karlotoxins can no longer be eluted and are effectively rendered bio-unavailable. (A. Place, pers. comm., October, 2016).

The removal efficiency of extracellular *K. veneficum* and *K. mikimotoi* ichthyotoxins proved to be algal species and clay type specific. As observed during our previous work with the haptophyte *Prymnesium parvum* (Seger et al. 2015; Chapter 2), non-swelling clay minerals, such as Korean loess, zeolite and kaolin proved poorly suited for removal of ichthyotoxins. In case of *K. mikimotoi*, treatment with kaolin completely mitigated cytotoxic effects, albeit only at concentrations five times higher than required for bentonite clay, which completely eliminated ichthyotoxicity at a clay loading of only 0.1 g L⁻¹. Bentonite type clays have

previously been shown to remove a wide range of dissolved algal toxins, such as the polyether ladder brevetoxin (Pierce et al., 2004), galactolipid prymnesins (Sengco et al., 2005) and peptide microcystins (Morris et al., 2000; Prochazka et al., 2013), yet these studies only focused on single bentonite types.

Screening of six different bentonite clays for *K. veneficum* ichthyotoxin removal revealed that the here assayed bentonites could be separated into two distinct groups: (1) those that performed poorly (B1-B3) and (2) those that achieved excellent removal of *K. veneficum* ichthyotoxicity (B4-B6). The difference in ichthyotoxin adsorption between these two clay groups could not be simply explained by clay particle size. Comparable particle size spectra obtained through sonication did not increase ichthyotoxin adsorption by B1-B3, although performance of B4 was much improved. This indicates that within group 2 (effective adsorbents), finer bentonite products were better suited for ichthyotoxin adsorption, while at the same time suggesting that the difference between the two groups is governed by intrinsic clay properties. We previously attributed differences in *P. parvum* ichthyotoxin adsorption by various bentonites to differences in zeta potential (clay surface charge) and montmorillonite content (Seger et al. 2015, Chapter 2). However, both cation exchange capacity (indicative of clay charge) and montmorillonite content did not correlate with *K. veneficum* ichthyotoxin removal (Table 4.1). Karlotoxin adsorption also proved independent of the major cation present (Na^+ or Ca^{++}), suggesting that cation specific surface-bridging as proposed by Figueroa et al. (2004) for adsorption of antibiotics, did not play a role. We did, however, observe a distinct difference in the swell index between the two bentonite groups. Poor *K. veneficum* adsorbents were characterised by a low swell index (7-12 mL/2g), whereas high ichthyotoxin adsorption correlated with a high swell index (20-24 mL/2g). As bentonite expands upon contact with water, previously inaccessible spaces between clay layers become available, thereby significantly increasing the clays' reactive surface area and allowing for intercalation of organic compounds into the hydrophobic interlayer space (Jahed et al., 2014; Lambri et al., 2010; Moyo et al., 2014; Santamarina et al., 2002; Yukselen and Kaya, 2008). The intercalation of organic molecules is dependent upon the extent of clay

swelling, as well as the charge and size of the adsorbate molecule (Carrasquillo et al., 2008; Jiang et al., 2002; Moyo et al., 2014; Wu et al., 2013). Since non-swelling kaolin and zeolite proved unsuitable for karlotoxin removal, it appears likely that karlotoxin adsorption by bentonite type clays predominantly occurs through intercalation. The amphipathic nature of the karlotoxin molecule (Van Wagoner et al., 2008), likely facilitated its adsorption in the hydrophobic interlayer space of B4-B6, while its relatively large molecular size prevented it from entering the considerably smaller interlamellar spaces of low-swelling bentonites B1-B3. Interpretation of the adsorption mechanism of *Karenia mikimotoi* ichthyotoxins is complicated by their unknown chemical nature. The ability of non-swelling kaolin to remove *K. mikimotoi* ichthyotoxicity suggests the involvement of external clay surfaces in the adsorption process.

4.5 Conclusions

We here successfully extended the application of clay minerals to completely remove extracellular *Karlodinium veneficum* and *Karenia mikimotoi* ichthyotoxins through direct adsorption onto clay particles. Ichthyotoxin adsorption proved clay type and algal species specific, with bentonite type clays outperforming Korean loess or zeolite and kaolin clays. However, not all bentonites assessed here could effectively adsorb *K. veneficum* ichthyotoxins. While cation exchange capacity, major cation and purity of bentonites poorly correlated with ichthyotoxin removal, the capacity of clays to swell proved critical. Selecting bentonites with a high swell index (>20 mL/2g) and finer particle size (<5 μ m) will improve treatment success. Complete elimination of *K. veneficum* and *K. mikimotoi* toxicity towards the highly sensitive gill cell line RTgill-W1 by fine bentonites could be achieved at concentrations as low as 0.1 g L⁻¹. Such low minimal effective clay loadings not only dramatically reduce the cost of application, but may help in clay dispersal becoming more acceptable in regions with high regulations on sediment additions. The application of clay for *K. veneficum* and *K. mikimotoi* ichthyotoxin adsorption offers great promise as a mitigation tool.

Chapter 5

Application of clay to mitigate reactive oxygen species mediated ichthyotoxicity by raphidophyte microalgae

Abstract

The variable nature of reactive oxygen species (ROS) mediated ichthyotoxicity by microalgae belonging to the Raphidophyceae presents a formidable challenge to mitigation efforts. We here explored the use of clay as both flocculant and ichthyotoxin adsorbent for the fish-killers *Chattonella marina*, *Heterosigma akashiwo* and *Fibrocapsa japonica*. Excellent ichthyotoxin removal efficiencies could be achieved with bentonite clay, with the timing of clay dosing proving critical. With lysed algal preparations, only partial removal of toxicity (~20% for all species) could be achieved, whereas direct application of clay during the process of cell lysis (as would occur in the field) completely eliminated *C. marina* and *H. akashiwo* toxicity. Adsorption experiments with the free fatty acid eicosapentaenoic acid (EPA) and putative fatty acid aldehyde lipid peroxidation end-products deca- and heptadienal, suggest that the direct application of clay during cell lysis rapidly removes fatty acids before they can undergo lipid peroxidation to non-absorbable end-products. Stored EPA (~6 months) exhibited higher toxicity towards the gill cell line than freshly prepared EPA (compare $4.0 \pm 1\%$ gill cell viability by $50 \mu\text{g mL}^{-1}$ fresh EPA vs. $17 \pm 3\%$ by $5 \mu\text{g mL}^{-1}$ old EPA). Both synthetic amino-clays and clays modified with polyaluminium chloride (PAC) achieved excellent *C. marina* cell (100% at 0.1 g L^{-1}), as well as ichthyotoxin removal. Polyaluminium chloride by itself (without clay) already completely eliminated *C. marina* toxicity towards the rainbow trout RTgill-W1 cell line and could dramatically improve the performance of non-adsorbent Korean loess (100% toxicity removal at 0.25 g L^{-1}). Clay application proved highly successful in reducing toxicity of all three ROS producing raphidophytes studied.

5.1 Introduction

The class Raphidophyceae comprises some of the most notorious fish-killing algal species, among them *Fibrocapsa japonica*, *Heterosigma akashiwo* and most notably *Chattonella marina*. The latter two species in particular have been the subject of intensive study, due to their devastating economic impact on global aquaculture operations (Chang et al., 1990; Hallegraeff et al., 1998; Imai et al., 2006). However, like many other ichthyotoxic algal species, the exact chemical nature of the compound(s) responsible for mass fish mortalities has remained elusive. One feature in common among all raphidophyte microalgae is that they are fragile cells that readily lyse even upon impact of the gills of fish. While previous work suggested the production of neurotoxic compounds by *H. akashiwo* and *F. japonica* (Khan et al., 1996; Khan et al., 1997) and/or involvement of extracellular organics (Twiner et al., 2005; Twiner et al., 2004), an increasing body of evidence points towards the involvement of reactive oxygen species (first proposed by Oda et al. (1997) and further explored by Dorantes-Aranda et al., 2013; Imai and Yamaguchi, 2012; Marshall et al., 2005). While not produced in sufficient quantities to explain observed fish mortalities on their own, reactive oxygen species (ROS) are thought to serve as cofactors of polyunsaturated fatty acid (PUFA) toxicity by promoting lipid peroxidation (Marshall et al., 2003; Marshall et al., 2005). Lipid peroxidation proceeds via a complex series of chemical reactions involving superoxide anions (O_2^-), H_2O_2 and potential other unidentified ROS, to yield a range of highly toxic, chemically diverse end-products, such as fatty acid aldehydes (Dorantes-Aranda et al., 2013; Eritsland, 2000; Halliwell and Gutteridge, 2015). While this process has been best described for *C. marina*, both *H. akashiwo* and *F. japonica* have also been demonstrated to produce significant levels of ROS (Dorantes-Aranda et al., 2015; Nakamura et al., 1998; Oda et al., 1997), as well as substantial amounts of PUFAs (47-55% of total fatty acids), such as eicosapentaenoic acid (EPA: 17-23% total fatty acids across all three species, Dorantes-Aranda et al., 2013; Fu et al., 2004; Marshall et al., 2002b).

The complex nature of the ichthyotoxic principle presents a formidable challenge to mitigation efforts. Clay, particularly bentonites, have been demonstrated in the

previous chapters to be an excellent adsorbent of *Prymnesium parvum* (Chapter 2), *Karlodinium veneficum* and *Karenia mikimotoi* ichthyotoxins (Chapter 3), yet it is impossible to predict how clay may interact with the chemically diverse precursors, intermediates and end-products of ROS mediated lipid peroxidation. In the past, clay minerals have been demonstrated to adsorb a wide range of chemical compounds (Gao and Pedersen, 2005; Henrichs and Sugai, 1993; Moyo et al., 2014; Polati et al., 2006; Putra et al., 2014). Clays often contain significant amounts of organic matter on their surface (Haynes and Naidu, 1998) that may serve as an alternative sink for ROS. Reactive oxygen species have also been proposed to play a role in the ichthyotoxicity of *K. mikimotoi* (Yamasaki et al., 2008), which could be completely removed by addition of either bentonite or kaolin clay (Chapter 3).

Sengco et al. (2001) demonstrated conventional clays to be well suited for removal of *H. akashiwo* cells (90% removal at 0.25 g L⁻¹), yet larger (60-80 µm) *C. marina* cells were not easily removed (Wu et al., 2010). However, excellent cell removal efficiencies can be achieved through modification of conventional clays with polyaluminium chloride (PAC), a flocculant commonly used in drinking water treatment (Pierce et al., 2004; Sengco and Anderson, 2004; Sengco et al., 2001). Additionally, synthetic amino-clays have been newly designed to lyse harmful algal cells (Lee et al., 2013b). The latter improves cell removal, but at the same time risks release of intracellular PUFAs and ROS. The implications of this for mitigation of overall ichthyotoxicity have not previously been assessed.

We here screened representatives of the four major clay groups, kaolin, zeolite, Korean loess and bentonite for removal of toxicity of lysed preparations of the raphidophyte algae *Chattonella marina*, *Heterosigma akashiwo* and *Fibrocapsa japonica*. Based on the well-studied ROS mediated ichthyotoxic principle of *C. marina*, we also conducted adsorption experiments with commercial preparations of eicosapentaenoic acid and the fatty acid aldehydes decadienal and heptadienal. Cell removal efficiency, as well as ichthyotoxin adsorptive capacity of polyaluminium chloride modified clays and synthetic amino-clays were assessed with the RTgill-W1 gill cell line assay (Dorantes-Aranda et al., 2011).

5.2 Methods

5.2.1 Algal cultures

Chattonella marina strain CMN-118 (Japan), *Heterosigma akashiwo* strain HAGB01 (Tasmania, Australia) and *Fibrocapsa japonica* strain FJCS332 (Japan) were grown in 250 mL glass flasks containing 150 mL of filtered coastal seawater (0.22 µm) at a salinity of 30. The media was enriched with GSe nutrients and cultures maintained at 20°C with light supplied at 120 µmol m⁻² s⁻¹ with a 12:12 h light dark cycle.

5.2.2 Clay material

Kaolin (Snobrite C), zeolite (ANZ38), Korean Loess (B) and bentonite clays (Ed) were screened through a 62 µm mesh to obtain a more uniform size fraction and subsequently suspended in deionised water before treatment of algal preparations. Detailed physicochemical properties of these clays are provided in Chapter 2, section 2.1.

5.2.3 Clay screening

Algal cultures were harvested during the late exponential/early stationary phase and lysed via sonication of 20 mL of culture on ice with a probe type sonicator (Measuring & Scientific Equipment Ltd., London, England) at an intensity of 7 µm peak-peak (Table 5.1). The efficiency of sonication was verified through cell counts (Coulter Counter, Beckman Z4). The pH of all lysates was adjusted to 8.5 before transferring 980 µL to 1.5 mL Eppendorf centrifuge tubes containing 20 µL of either concentrated kaolin, zeolite, Korean loess or bentonite clay slurry that yielded final clay concentrations of 0-0.5 g L⁻¹. Controls received 20 µL of respective clay slurry or deionised water added to 980 µL of Gse medium (pH 8.5). Samples were vortexed for 5 s on high and centrifuged at 9300 x g for 1 min. The resulting supernatant was tested on the gill cell line RTgill-W1 in quadruplicate wells of trans-well permeable 96-well plates (Corning 3381) during 2 h exposures in the light (120 µmol m⁻² s⁻¹). The assays are described in detail in Chapter 2, section 2.4.

In an additional experiment, 60 µL of bentonite clay (0-0.5 g L⁻¹) was added to 2.94 mL of *H. akashiwo* (135,000 cells mL⁻¹) or *C. marina* culture (29,000 cells mL⁻¹) in 15

mL centrifuge tubes immediately prior to sonication (30 s at amplitude of 7 μm peak to peak). Resulting clay-algal lysate suspensions were centrifuged at 1400 x g (Hettich Universal 16A) and the supernatant tested on the gill cells. *Fibrocapsa japonica* strain FJSC332 was lost in culture before these experiments could be conducted.

Table 5.1 Preparation of algal lysates for clay screening experiments.

Species	Cell concentration (cells mL ⁻¹)	Sonication time (min)	Sonication efficiency (% lysed cells)
<i>Chattonella marina</i>	15,000	2	98.5
<i>Heterosigma akashiwo</i>	90,000	5	98.7
<i>Fibrocapsa japonica</i>	60,000	10	95.7

5.2.4 Mechanism of ichthyotoxin adsorption

5.2.4.1 Lipid peroxidation precursors and end-products

Commercial preparations of eicosapentaenoic acid (EPA; E2011, Sigma), decadienal (180513, Sigma) and heptadienal (180548, Sigma) dissolved in methanol were diluted in GSe medium (pH 8.5) to yield final concentrations of 50, 3 and 15 $\mu\text{g mL}^{-1}$, respectively. An additional solution of 5 $\mu\text{g mL}^{-1}$ EPA stored at -20 °C for ~6 months was also prepared. The final methanol concentration in GSe was 1% (v/v). All four solutions were treated either with conventional bentonite clay (Ed) or bentonite of smaller particle size prepared by sonication of one mL of clay slurry for 40 min at 7 μm peak-peak. The adsorption experiments were conducted in 1.5 mL Eppendorf tubes and followed the protocol described above for algal culture lysates (see 2.3).

5.2.4.2 Reactive oxygen species

Production of superoxide by *C. marina* was measured following the protocol of Godrant et al. (2009). Results are expressed as superoxide production rate (pmol cell⁻¹ h⁻¹) obtained by comparing production of sample to a standard curve obtained with the xanthine (0.5 mM) / xanthine oxidase (0.1, 0.7 and 1.5 U L⁻¹) system. To measure the effect of clay application on superoxide production, bentonite clay (0-0.5 g L⁻¹) was added directly to *C. marina* culture during sonication (see 2.3). Superoxide production in the resulting supernatant was detected through the

addition of 10 μL of 125 μM MCLA (87787, Sigma) to 270 μL of sample.

Luminescence of MCLA in presence of superoxide was quantified in a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413–3350) and corrected with a blank consisting of clay treated sample (0-0.5 g L^{-1}) spiked with 5 kU mL^{-1} of superoxide dismutase (S7571, Sigma). Results are expressed as blank corrected chemiluminescence units.

To test whether organic materials present on the clay surface influenced ichthyotoxin adsorption, conventional bentonite, Korean loess and kaolin were treated with H_2O_2 . To remove organic matter, 5 g of clay sample were added to 500 mL glass beakers and resuspended in 100 mL of 3% H_2O_2 . The sample was heated to 60°C on a hotplate and upon cessation of bubbling additional increments of 30 mL of H_2O_2 added. Organic matter was considered removed when further additions of H_2O_2 did not restart bubbling. At this stage, samples were evaporated to dryness and resuspended in deionised water. No residual H_2O_2 was detected with the broadly used iodide-acetic acid method. Thus treated, as well as conventional clays were subsequently tested for removal of *C. marina* toxicity towards the gill cell line following the protocol described above for direct application of clay during sonication (see 2.3).

5.2.5 Modified clay

5.2.5.1 Cell removal

Cell removal experiments followed the protocol outlined in Chapter 2, section 2.3.1. Briefly, clay was suspended in deionised water, such that the addition of 2 mL to 30 mL of *C. marina* culture (20,000 Cells mL^{-1}) yielded final concentrations of 0-0.5 g L^{-1} (3 replicates per concentration). Controls received 2 mL of deionised water and all samples were allowed to settle for 3 h. Cell concentration in the overlying 25 mL was enumerated through Coulter Counter cell counts (Beckman, Z4) and the percentage cell removal efficiency calculated. The clays thus assayed included conventional, untreated kaolin and bentonite, as well as synthetic Fe-, Mg-, and Ca-amino-clays (Lee et al., 2013b). For polyaluminium chloride (PAC, Telford Industries) modified clays, preliminary experiments indicated a PAC to clay ratio of 0.4:1 best

suites for *C. marina* cell removal (data not shown). Both conventional Korean loess and bentonite were thus modified and tested with the above described protocol.

5.2.5.2 Ichthyotoxin adsorption

Korean Loess and bentonite clays were modified with PAC as described above for cell flocculation experiments (2.5.1) and applied to *C. marina* cultures directly during cell lysis following established protocol (see 2.3). To test for the effect of PAC alone (no clay), an additional treatment consisted of pure PAC at concentrations identical to those employed to modify clays (0.4 times clay loading).

5.2.6 Data analysis

All values given represent the mean \pm 1 standard deviation. All graphs were designed with the statistical package R (www.r-project.org).

5.3 Results

5.3.1 Clay screening

Challenging the gill cell line RTgill-W1 with lysed preparations of *Fibrocapsa japonica*, *Heterosigma akashiwo* and *Chattonella marina* cultures significantly reduced gill cell viability down to 64 ± 3 , 39 ± 3 and $23 \pm 5\%$, respectively (Fig. 5.1). Application of clay to these preparations improved gill cell viability by an average of 20% across all species, with the reduction in cytotoxicity proving dependent upon the type of clay used. While zeolite, kaolin and Korean loess all increased viability of gill cells exposed to *H. akashiwo* and *F. japonica*, only bentonite proved suitable in the case of *C. marina*. Bentonite type clay consistently performed best, but cytotoxicity from lysed algal preparations could not be completely eliminated. In the case of *F. japonica* (Fig. 5.1a) and *H. akashiwo* (Fig. 5.1b), no further increases in gill cell viability were observed with clay loadings exceeding 0.25 g L^{-1} , while for *C. marina*, the maximum beneficial effect (20% gill cell viability increase) of clay application occurred at 0.1 g L^{-1} (Fig. 5.1c). Higher clay loadings did not further improve gill cell viability unless applied during cell lysis (Fig. 5.1d). In this case, application of bentonite clay succeeded in completely eliminating *C. marina* and *H. akashiwo* toxicity towards the gill cell line at clay loadings as low as $0.1\text{--}0.25 \text{ g L}^{-1}$.

Fibrocapsa japonica FJCS332 was lost in culture during this work and could not be included in these further experiments.

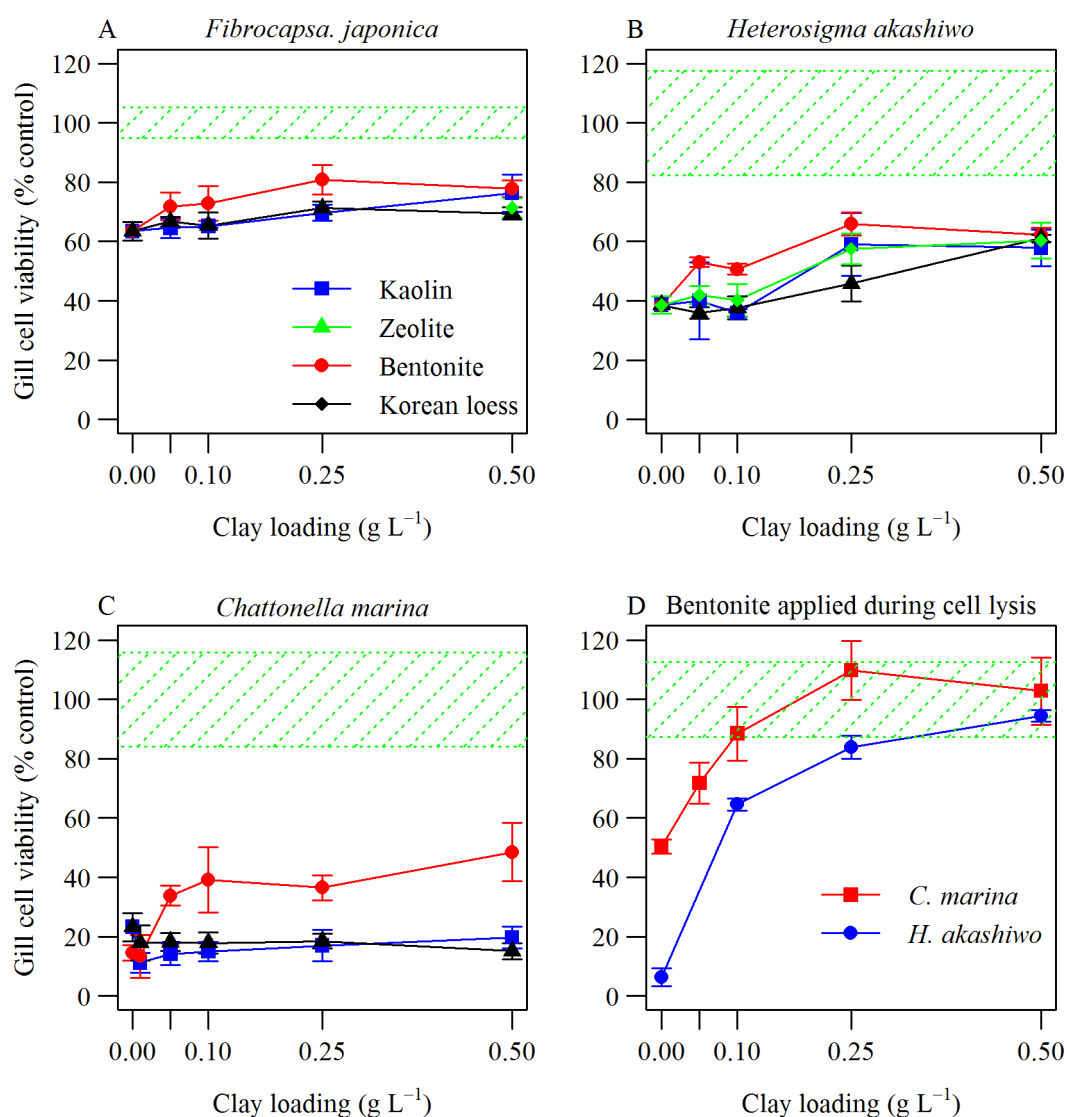


Figure 5.1 Viability of rainbow trout RTgill-W1 cells exposed to lysed preparations of *F. japonica* (A), *H. akashiwo* (B) and *C. marina* (C) treated with 0-0.5 g L⁻¹ of kaolin, zeolite, Korean loess or bentonite clay. (D) Direct application of bentonite clay during lysis of *C. marina* and *H. akashiwo* cells (*F. japonica* could not be included due to loss of strain in culture). Green shaded area indicates 1 standard deviation around the control.

5.3.2 Mechanism of ichthyotoxin adsorption

Exposure of gill cells to commercial fatty acid and fatty acid aldehyde preparations revealed significant differences in gill cell viability after bentonite clay treatment (Fig. 5.2). Toxicity of fresh EPA preparations towards the gill cell line was readily removed through clay application, with sonicated clay performing better than conventional clay (Fig. 5.2a and b). Stored (6 months) EPA proved orders of magnitude more toxic than fresh EPA (Fig. 5.2a, reduction of $4.0 \pm 1\%$ gill cell viability by $50 \mu\text{g mL}^{-1}$ fresh EPA vs. $17 \pm 3\%$ by $5 \mu\text{g mL}^{-1}$ old EPA). Toxicity of this older EPA preparation could only partially be removed, with clay concentrations exceeding 0.1 g L^{-1} not resulting in further gill cell viability increases. High toxicity of the fatty acid aldehydes decadienal ($62.3 \pm 7.7\%$ gill viability at $3 \mu\text{g mL}^{-1}$) and heptadienal ($39 \pm 13.1\%$ at $15 \mu\text{g mL}^{-1}$) could not be removed through application of either conventional or sonicated bentonite (Fig. 5.2c and d).

Removal of organic matter via hydrogen peroxide (H_2O_2) treatment of clay before application to *C. marina* during cell lysis did not significantly improve viability of gill cells when compared to untreated, conventional clays (Fig. 5.3a). Only bentonite type clay achieved complete removal of cytotoxicity, with Korean loess and Kaolin only marginally increasing gill cell viability. Lysed and centrifuged preparations of *C. marina* prepared from stationary phase cultures were measured to produce $3.15 \pm 0.28 \text{ pmol cell}^{-1} \text{ h}^{-1}$ of superoxide (data not shown). Treatment of these preparations with bentonite clay did not affect superoxide production (presented as chemiluminescence units) in the concentration range of $0\text{--}0.5 \text{ g L}^{-1}$ (Fig. 5.3b).

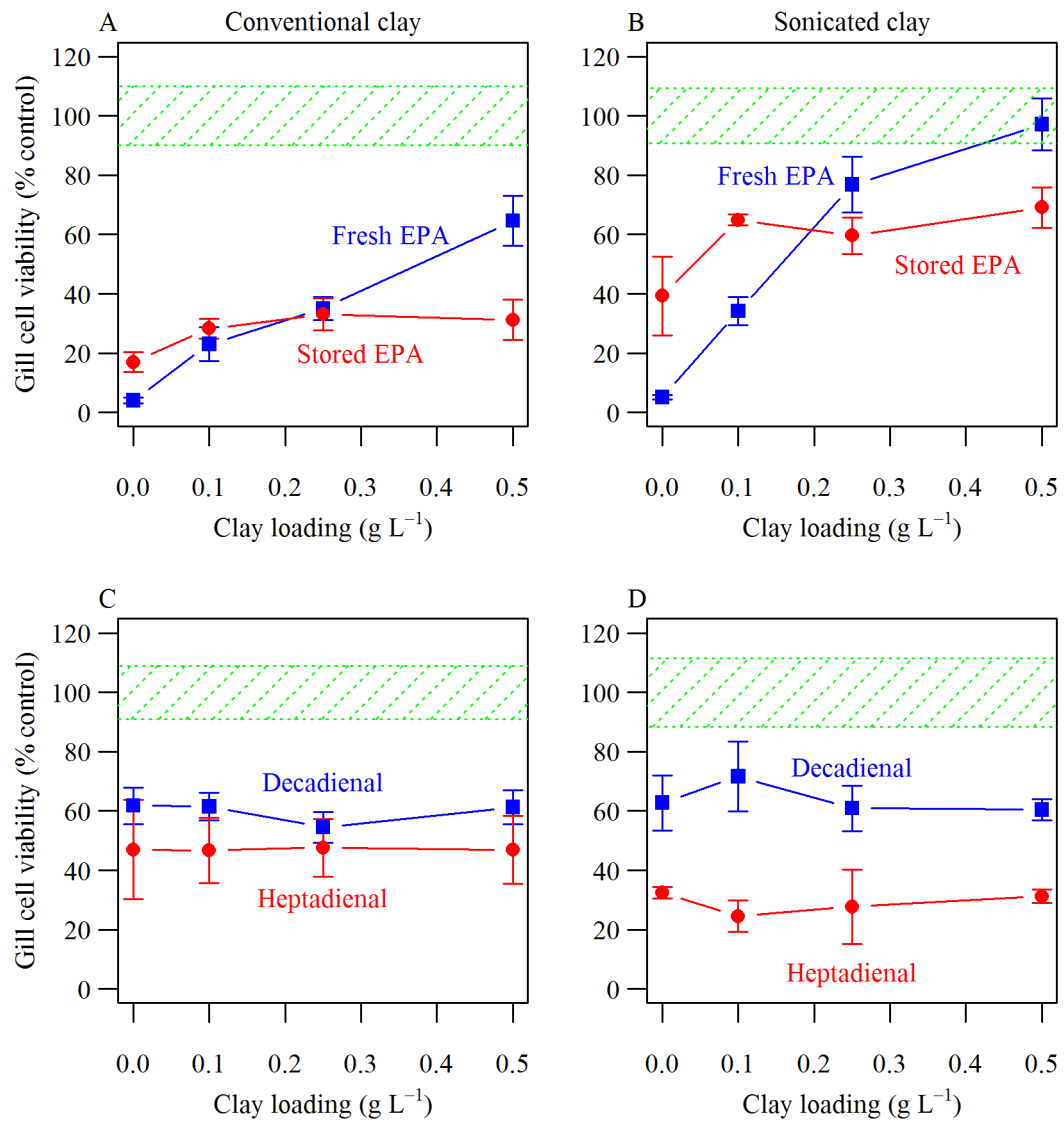


Figure 5.2 Gill cell viability after exposure to commercial eicosapentanoic acid (EPA), decadienal or heptadienal preparations treated with bentonite clay. Fresh and stored (6 months) EPA treated with conventional (A) or sonicated bentonite (B). Decadienal and heptadienal treated with either conventional (C) or sonicated bentonite (D). Green shaded area represents 1 standard deviation around the non-toxic control.

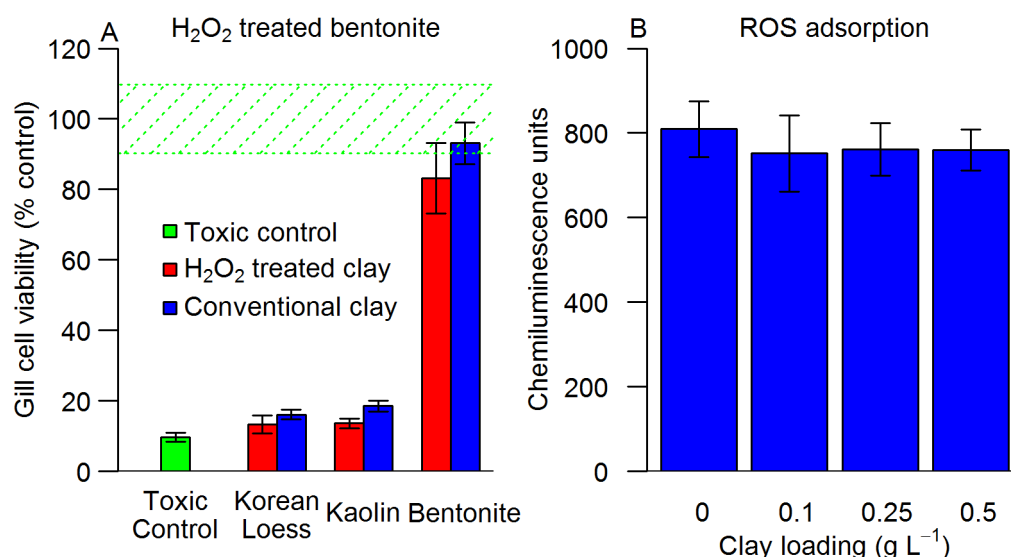


Figure 5.3 (A) Gill cell viability after exposure to *C. marina* preparations treated with either conventional or hydrogen peroxide (H₂O₂) treated clays during cell lysis. (B) Reactive oxygen species production (measured as chemiluminescence of MCLA probe) of lysed *C. marina* culture.

5.3.3 Modified clay

5.3.3.1 Flocculation

Conventional kaolin and bentonite type clays proved unsuitable for *C. marina* cell removal purposes, only achieving a maximum removal efficiency of 20% at the highest clay loading of 0.5 g L⁻¹ (Fig. 5.4a). However, modification of bentonite or Korean loess with polyaluminium chloride (PAC) significantly improved cell removal efficiency compared to conventional clays, with clay concentrations as low as 0.1 g L⁻¹ removing 99% of *C. marina* cells (Fig. 5.4b). Synthetic amino-clays achieved equally high cell removal efficiencies, with Fe-modified aminoclay proving better suited for cell removal purposes than Mg- or Ca-aminoclay (Fig. 5.4c).

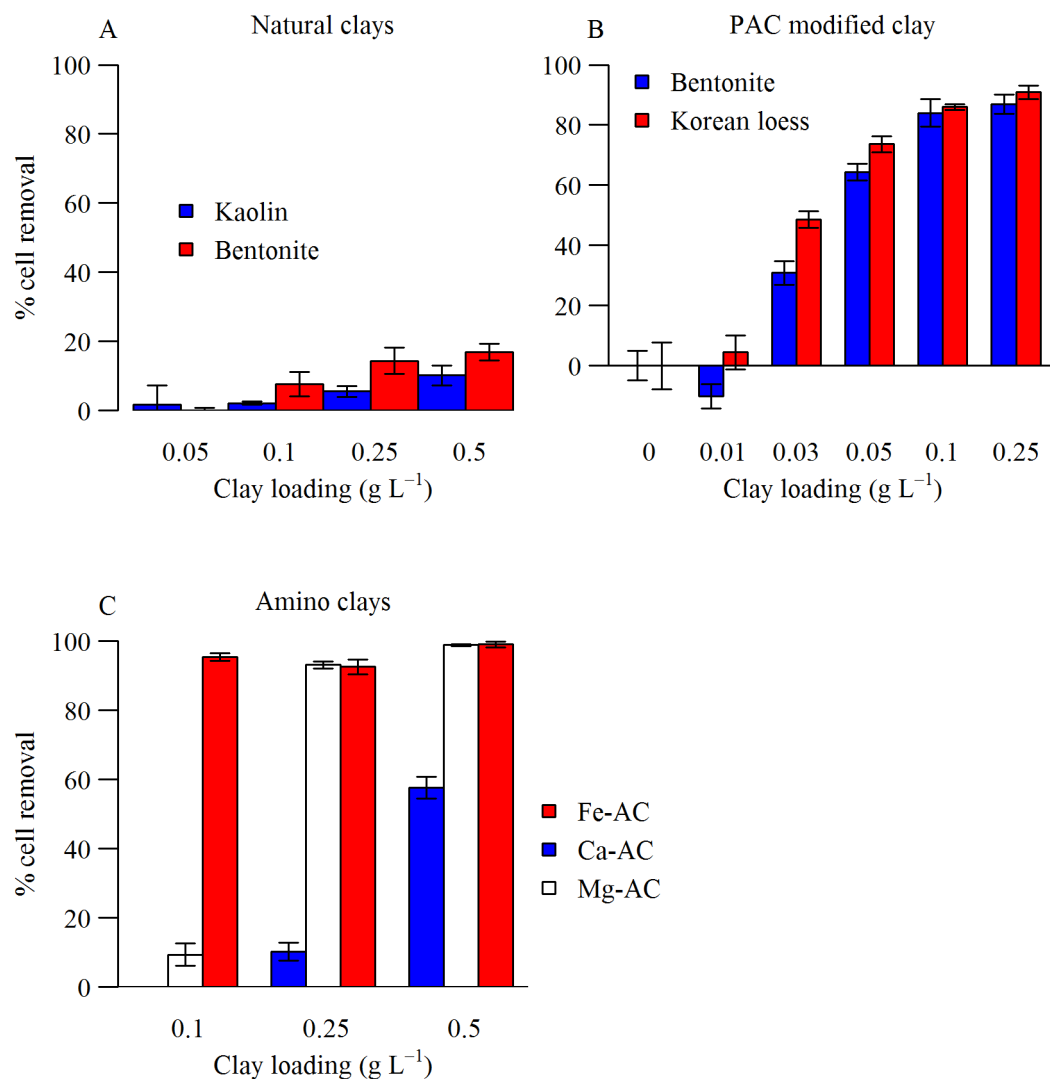


Figure 5.4 *Chattonella marina* cell removal efficiency by (A) conventional clays, (B) polyaluminium chloride (PAC) modified clays and (C) synthetic aminoclays.

5.3.3.2 Ichthyotoxin adsorption

Modification of Korean loess and bentonite type clay with PAC completely eliminated toxicity of lysed *C. marina* preparations towards the gill cell line (Fig. 5.5a). Polyaluminium chloride itself completely removed *C. marina* toxicity at concentrations equal to those used for modifying the clay materials (0.4 x clay concentration). Even Korean loess, which previously exhibited poor removal of toxicity, proved highly effective in combination with PAC. Treatment of identical

culture preparations with Fe-AC also completely eliminated *C. marina* toxicity towards the gill cell line at clay loadings of 0.25 g L⁻¹ (Fig. 5.5b).

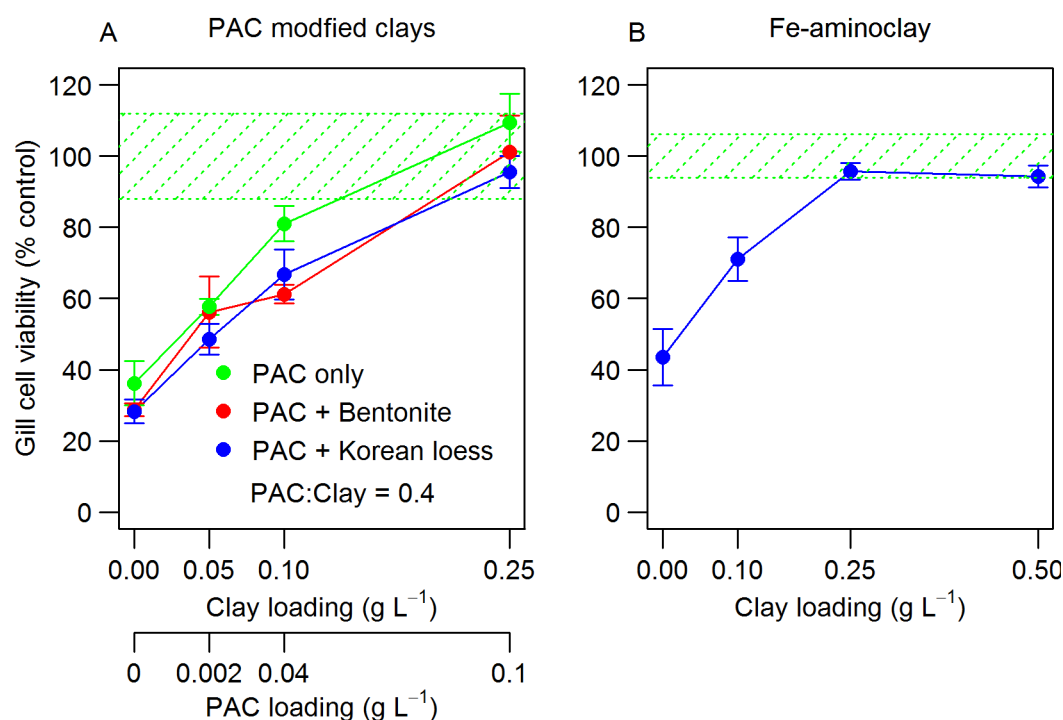


Figure 5.5 Gill cell viability after exposure to *C. marina* preparations treated with either PAC modified bentonite, Korean loess, pure PAC (A) or Fe-aminoclay (B) during cell lysis. Pure PAC concentration was 0.4 times that indicated by clay loading.

5.4 Discussion

Screening of four different clay types against three fish-killing raphidophyte algae, demonstrated that clay minerals are effective adsorbents of *Fibrocapsa japonica*, *Heterosigma akashiwo* and *Chattonella marina* ichthyotoxins. Employing the highly sensitive gill cell line assay in concert with direct measurements of superoxide production (*cf.* Dorantes-Aranda et al., 2015) allowed us to interpret the ichthyotoxin adsorptive behaviour of bentonite clay.

As previously demonstrated for *Prymnesium parvum* (Chapter 2) and *Karlodinium veneficum* ichthyotoxins (Chapter 3), bentonite type clays proved to be best suited for ichthyotoxin removal, completely eliminating toxicity of *H. akashiwo* and *C.*

marina. The timing of clay dosing proved critical, however. Unlike extracellular *K. veneficum*, *K. mikimotoi* and *P. parvum* ichthyotoxins that could be readily adsorbed by bentonite clay, we only achieved partial removal of toxicity in lysed preparations of *F. japonica*, *H. akashiwo* and *C. marina*. However, when clay was applied during the process of cell lysis, complete removal of *H. akashiwo* and *C. marina* toxicity occurred. This difference in adsorption success during or after cell lysis points to the presence of chemically distinct cytotoxic compounds generated by lipid peroxidation (Dorantes-Aranda et al., 2013; Halliwell and Gutteridge, 2015).

Lysis of *C. marina* cells dramatically increases production of superoxide anion (Dorantes-Aranda et al., 2015), a highly reactive oxygen species (ROS). Although not sufficient to cause lipid peroxidation on its own, its acid form HO_2^\bullet can drive lipid peroxidation, albeit at very low rates (Bielski et al., 1983). Superoxide anion is thought to yield highly reactive hydroxyl radicals (OH^\bullet) in the presence of transition metals via the Fenton reaction (often misrepresented as Haber-Weiss type reactions, see Koppenol, 2001). This radical (OH^\bullet) was proposed to initiate lipid peroxidation, but could not be detected in *C. marina* cultures with a recently developed, highly sensitive chemiluminescent assay (Miller et al., 2011, C. J. Miller, pers. comm., November 2016). It appears likely that other, yet unidentified ROS react with fatty acids (EPA in case of *C. marina*) to yield several highly toxic compounds, such as fatty acid aldehydes (Bruna et al., 1989; Dorantes-Aranda et al., 2013; Eritsland, 2000). Adsorption experiments with pure preparations of these aldehydes (deca- and heptadienal) and *C. marina*'s major fatty acid, EPA (20-23% total fatty acids, Marshall et al., 2002b), showed that only the fatty acid precursor can effectively be removed through clay application. Even smaller (ground) clay particles, which increased the removal of EPA, could not improve decadienal or heptadienal removal. Furthermore, we could only achieve partial adsorption of toxicity of old EPA (~6 months) subject to lipid peroxidation even during cold storage (Bruna et al., 1989; Chaijan et al., 2006; Eritsland, 2000). We interpret this to mean that the direct application of clay during cell lysis removes fatty acids (EPA) before they can undergo lipid peroxidation to non-absorbable end-products, such as fatty acid aldehydes. Against expectation, direct interaction between clay and

ROS did not appear to play a role, as pre-treatment of clay with H₂O₂ to remove organic matter on the clay surface did not alter the ichthyotoxin adsorptive profile and no reduction in O₂⁻ production after clay treatment was observed.

While we could demonstrate complete removal of *C. marina* and *H. akashiwo* toxicity by direct clay application during cell lysis, *F. japonica* strain (FJCS332) was lost in culture before any further experiments could be conducted. Various compounds have been proposed to form part of *H. akashiwo* and *F. japonica*'s ichthyotoxic principles (de Boer et al., 2009; Fu et al., 2004; Khan et al., 1996; Khan et al., 1997; Ling and Trick, 2010; Twiner et al., 2005), yet in the present work these species displayed remarkably similar toxin adsorption profiles to *C. marina*. All three species are well known producers of reactive oxygen species (Dorantes-Aranda et al., 2015; Nakamura et al., 1998; Oda et al., 1997) as well as peroxidisable polyunsaturated fatty acids, such as EPA (Dorantes-Aranda et al., 2013; Fu et al., 2004; Marshall et al., 2002b).

The effectiveness of clay application could be greatly increased through the addition of polyaluminium chloride (PAC). Originally employed to promote the flocculation of harmful algal cells (Pierce et al., 2004; Sengco and Anderson, 2004; Sengco et al., 2001), its potential to remove ichthyotoxins had not previously been assessed. We here demonstrated PAC treatment to not only significantly improve *C. marina* cell removal, but to also completely eliminate toxicity towards the gill cell line. Ineffective for flocculation purposes on its own, PAC requires the co-application of denser clay particles for sedimentation to occur (Lürling and van Oosterhout, 2013; Sengco et al., 2001). However, PAC alone (without clay) could also completely remove *C. marina* toxicity, suggesting that clay types other than bentonite could achieve high removal. Modifying poorly adsorbing conventional Korean loess with PAC thus proved successful in achieving complete removal of *C. marina* toxicity (Fig. 5.5). Synthetic amino-clays designed to serve as lytic agents of algal cells proved equally well suited for both cell and ichthyotoxin removal. Despite causing extensive cell lysis (100% cell removal at 0.25 g L⁻¹), Fe-aminoclay completely eliminated *C. marina* toxicity (Fig. 5.5). This indicates rapid adsorption of *C. marina* ichthyotoxins upon release from algal cells. While manipulations in the timing of

clay addition provided crucial insight into the role of ROS, the ability of clay to rapidly scavenge lipid peroxidation precursors will no doubt prove critical during clay application to fragile raphidophytes in the field.

5.5 Conclusions

Application of clay to the ROS producing raphidophyte species *Heterosigma akashiwo*, *Fibrocapsa japonica* and *Chattonella marina* proved highly successful in reducing toxicity towards the gill cell line RTgill-W1. Bentonite type clays proved to be excellent adsorbents of toxicity, with the timing of clay dosing critical. In lysed algal preparations, only partial removal of toxicity (~20% for all species) was achieved, whereas direct application of clay during the process of cell lysis completely eliminated *C. marina* and *H. akashiwo* toxicity. Our adsorption experiments with the free fatty acid eicosapentaenoic acid (EPA) and putative fatty acid lipid peroxidation aldehyde products (deca- and heptadienal), suggest that the direct application of clay during cell lysis rapidly removes the parent fatty acids (EPA) before they undergo lipid peroxidation to non-absorbable end-products, such as deca- and heptadienal. In a field application scenario, clay would be directly applied to intact algal cells and complete adsorption of ichthyotoxins released by lysis of fragile algal cells expected. We newly obtained evidence on the benefit of employing modified or synthetic amino-clays (Lee et al., 2013b; Wu et al., 2010) in that their application extends beyond cell removal to include direct adsorption of *C. marina* ichthyotoxins. While denser clay particles are required for effective flocculation to occur, even poor ichthyotoxin adsorbents such as Korean loess could be rendered effective through PAC addition.

Chapter 6

Application of clay to mitigate fish-killing effects by *Alexandrium* dinoflagellates

Abstract

We screened four clay types for removal of ichthyotoxins produced by *Alexandrium minutum* and two strains from the *Alexandrium tamarense* complex (Chilean *A. catenella* and Australian *A. tamarense*) while simultaneously measuring superoxide production. Lysed preparations of all *Alexandrium* species proved highly toxic to the RTgill cell-line, reducing cell viability to 0-20% during 2 h exposures. Bentonite clay application (0.5 g L^{-1}) reduced *Alexandrium* cytotoxicity by 20-40% in lysed preparations, but could completely eliminate toxicity when applied directly during the process of lysis. High total superoxide anion production ($12\text{-}18 \text{ pmol cell}^{-1}\text{hr}^{-1}$) was detected in all three isolates, suggesting involvement in cytotoxicity of reactive oxygen species mediated lipid peroxidation. The flocculant polyaluminium chloride, synthetic Fe-aminoclay, kaolin, zeolite and Korean loess proved ineffective for removal of *A. catenella* toxicity. Only bentonite type clay could completely eliminate cytotoxic effects. Our findings highlight that careful selection of clay type is critical for successful mitigation of *Alexandrium* ichthyotoxicity. Similarities in ichthyotoxin adsorption profiles and ROS production between *Alexandrium* and the taxonomically unrelated raphidophytes are remarkable.

6.1 Introduction

The *Alexandrium* species complex ranks among the best studied harmful dinoflagellate groups due to the species specific production of Paralytic Shellfish Poisoning toxins (PSTs) and their adverse effects on human health (Anderson et al., 2012; Hallegraeff, 1993). Blooms of members of this genus not only present a severe risk to the shellfish industry, but have repeatedly been implicated in mass mortalities of finfish (Cembella et al., 2002; Mardones et al., 2010; Martin et al., 2006; Montoya et al., 1996). While the PST profiles of several *Alexandrium* spp. and individual strains within those species are chemically well characterised, their fish-killing mechanism is poorly understood.

Initial work claimed PSTs to be directly responsible for ichthyotoxic effects (Montoya et al., 1996; Sephton et al., 2007), yet more recent investigations suggest involvement of other, yet unidentified compounds with allelopathic and lytic properties (Arzul et al., 1999; Tang et al., 2007). Much of this work focused on algal and cell line bioassays to identify a range of lytic compounds that may potentially be involved in ichthyotoxicity (Emura et al., 2004; Ma et al., 2011; Yamasaki et al., 2008). However, lytic activity by several strains of *A. tamarense* was found to be unrelated to cellular PST content (Tillmann et al., 2016) and has been observed even in the absence of PST production (Tillmann and John, 2002).

Employing the highly sensitive gill cell line RTgill-W1 assay, Mardones et al. (2015) demonstrated that less than 30% of total *A. catenella* cytotoxicity could be explained by the PST analogues C1&C2, STX, GTX 1&4. Instead, reactive oxygen species (ROS) mediated lipid peroxidation of *A. catenella*'s dominant polyunsaturated fatty acid (docosahexanoic acid) was claimed to be largely responsible for observed ichthyotoxic effects. Docosahexanoic acid (DHA) is a dominant polyunsaturated fatty acids (PUFAs) in many dinoflagellate species (Dalsgaard et al., 2003) with high levels reported in *A. tamarense* (Hammann et al., 2013) which can also be a producer of the ROS H_2O_2 (Kim et al., 1999). It is therefore possible that the synergistic role of ROS and DHA and/or other PUFAs as driver of ichthyotoxicity could apply to other *Alexandrium* species.

Paralytic Shellfish Poisoning toxins, such as saxitoxin, can readily be adsorbed onto clay particles (Burns et al., 2009; Lu et al., 2015), yet their suitability for removal of apparently chemically distinct *Alexandrium* ichthyotoxins has not previously been assessed. If reactive oxygen species mediated lipid peroxidation were involved, very similar ichthyotoxin adsorption profiles to those described in the previous chapter for ROS producing raphidophyte algae would be expected (Chapter 5). That is, partial adsorption in lysed algal preparations and complete removal of toxicity when clay is applied directly during cell lysis would be expected.

To determine the effectiveness of clay to remove *Alexandrium* ichthyotoxins, we therefore screened four different clay types (kaolin, zeolite, Korean loess and bentonite) for removal of *Alexandrium catenella*, *A. tamarense* and *A. minutum* toxicity towards the gill cell line RTgill-W1. To interpret ichthyotoxin adsorption profiles, superoxide production was monitored and the capacity of clay to remove DHA toxicity tested. Further experiments investigated the suitability of polyaluminium chloride modified clay and synthetic amino-clay to remove *A. catenella* cytotoxicity.

6.2 Methods

6.2.1 Algal cultures

Alexandrium catenella strain DAVIS10 (Aysén, Chile; group 1), *A. tamarense* strain ATTR/F (Tasmania, Australia; group 1) and *A. minutum* strain AMCS323 (South Australia) were grown in 250 mL glass flasks containing 150 mL of filtered coastal seawater (0.22 µm) at a salinity of 30. The media was enriched with GSe nutrients and cultures maintained at 17°C with light supplied at 120 µmol m⁻² s⁻¹ with a 12:12 h light dark cycle. The average cell volume was determined with a Coulter Counter (Beckman, Z4) and PST profiles obtained from the literature (Table 6.1).

6.2.2 Clay material

Kaolin (Snobrite C), zeolite (ANZ38), Korean Loess (B) and bentonite clays (Ed) were screened through a 62 µm mesh to obtain a more uniform size fraction and subsequently suspended in deionised water before treatment of algal preparations.

Detailed physicochemical properties of these clays are provided in Chapter 2, section 2.1.

Table 6.1 Paralytic Shellfish Toxin profiles (pg cell⁻¹) and cell volumes of the three *Alexandrium* strains studied here.

Strain	DAVIS10 ^a	AMCS323 ^b	AT.TR/F ^b
Average cell volume (μm ³)	15,925	4,641	14,410
C1	52.38	-	49.75
C2	-	-	2254.61
C4	180.94	-	8.64
dcGTX3	-	-	2.94
GTX2	1047.00	10.85	5.2
GTX3	-	13.10	9.78
GTX1	504.53	9.19	107.19
GTX4	-	14.48	286.62
GTX5	603.17	-	-
GTX6	587.99	-	-
NEO	614.08	-	53.51
STX	72.5	-	-

^aAguilera-Belmonte et al. (2011)

^b(Dorantes-Aranda et al., 2017)

6.2.3 Clay screening

Alexandrium catenella (4740 cells mL⁻¹), *A. minutum* (27,000 cells mL⁻¹) and *A. tamarense* (2,740 cells mL⁻¹) cultures were harvested during the late exponential/early stationary phase and lysed via sonication of 20 mL of culture on ice with a probe type sonicator (Measuring & Scientific Equipment Ltd., London, England). Sonication times were as follows: 10 min (*A. catenella* and *A. tamarense*) and 30 min (*A. minutum*). Cell lysis was verified through microscopic inspection. The pH of all lysates was adjusted to 8.5 and 980 μL transferred to 1.5 mL Eppendorf centrifuge tubes containing 20 μL of either concentrated kaolin, zeolite, Korean loess or bentonite clay slurry to yield final clay concentrations of 0-0.5 g L⁻¹. Controls received 20 μL of respective clay slurry or deionised water added to 980 μL of GSe medium (pH 8.5). Samples were vortexed for 5 s on high and centrifuged at 9300 x g for 1 min. The resulting supernatant was tested on the gill cell line RTgill-W1 in

quadruplicate wells of trans-well permeable 96-well plates (Corning 3381) during 2 h exposures in the light ($120 \mu\text{mol m}^{-2} \text{s}^{-1}$). The assays are described in detail in Chapter 2, section 2.4.

In an additional experiment, 60 μL of bentonite clay ($0\text{--}0.5 \text{ g L}^{-1}$) was added to 2.94 mL of *A. catenella* ($12,000 \text{ cells mL}^{-1}$) culture in 15 mL centrifuge tubes immediately prior to sonication (45 s at 7 μm peak to peak). Resulting clay-algal lysate suspensions were centrifuged at $1400 \times g$ (Hettich Universal 16A) and the supernatant tested on the gill cells.

6.2.4 Mechanism of ichthyotoxin adsorption

6.2.4.1 Docosahexanoic acid adsorption

A commercial preparation of docosahexanoic acid (D2534, Sigma) dissolved in methanol was diluted in GSe medium (pH 8.5) to yield a final concentration of $7 \mu\text{g mL}^{-1}$ and 1% (v/v) methanol. The preparation was treated with conventional bentonite clay (Ed) to yield final concentrations of $0\text{--}0.5 \text{ g L}^{-1}$. Adsorption experiments were conducted in 1.5 mL Eppendorf tubes and followed the protocol described above for algal culture lysates (see 5.2.3).

6.2.4.2 Superoxide production

Production of superoxide by all three *Alexandrium* species was measured following the protocol of Godrant et al. (2009). Results are expressed as superoxide production rate ($\text{pmol cell}^{-1} \text{h}^{-1}$), obtained by comparing production of sample to a standard curve obtained with the xanthine (0.5 mM) / xanthine oxidase ($0.1, 0.7$ and 1.5 U L^{-1}) system. Superoxide dismutase (5 kU mL^{-1}) treated sample served as the blank. Superoxide was quantified by measuring luminescence of the probe MCLA (87787, Sigma) in a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413–3350).

6.2.5 Modified clays

Korean Loess and bentonite clays were modified with polyaluminium chloride (PAC) in a PAC to clay ratio of 0.4:1 and applied to *A. catenella* culture ($4600 \text{ cells mL}^{-1}$) directly during cell lysis following established protocol (see 2.3). To test for the

effect of PAC alone (no clay), an additional treatment consisted of pure PAC at concentrations identical to those employed to modify clays (0.4 times clay loading).

6.2.6 Data analysis

Reactive oxygen species production data were tested for normality and homogeneity of variance with Kolmogorov-Smirnov and Levene's tests, respectively. As indicated by these tests, the data was square root transformed to meet ANOVA assumptions. Resulting significant effects were followed up with Tukey's HSD. All values given represent the mean \pm 1 standard deviation. All statistical analysis and graphs were conducted with the statistical package R (www.r-project.org).

6.3 Results

6.3.1 Clay screening

All three species of *Alexandrium* assessed here proved highly toxic towards the gill cell line (Fig. 6.1), with lysed preparations of *A. catenella* and *A. minutum* cultures exhibiting the highest toxicity (no viable gill cells remained after 2 h exposure), closely followed by *A. tamarense* (20% of gill cells remained viable). Out of the four clay types tested (kaolin, zeolite, Korean loess and bentonite), bentonite performed best in terms of cytotoxicity reduction. Treatment of lysed preparations of *Alexandrium* spp. with bentonite significantly improved gill cell viability by up to 40%, but could not completely eliminate toxicity. This maximum beneficial effect was observed at a clay concentration of 0.1 g L⁻¹ (Fig. 6.1a-c), with higher clay loadings not resulting in any further viability increases. However, when bentonite clay was applied to *A. catenella* directly during the process of cell lysis, toxicity towards the gill cell line could be completely eliminated (Fig. 6.1d).

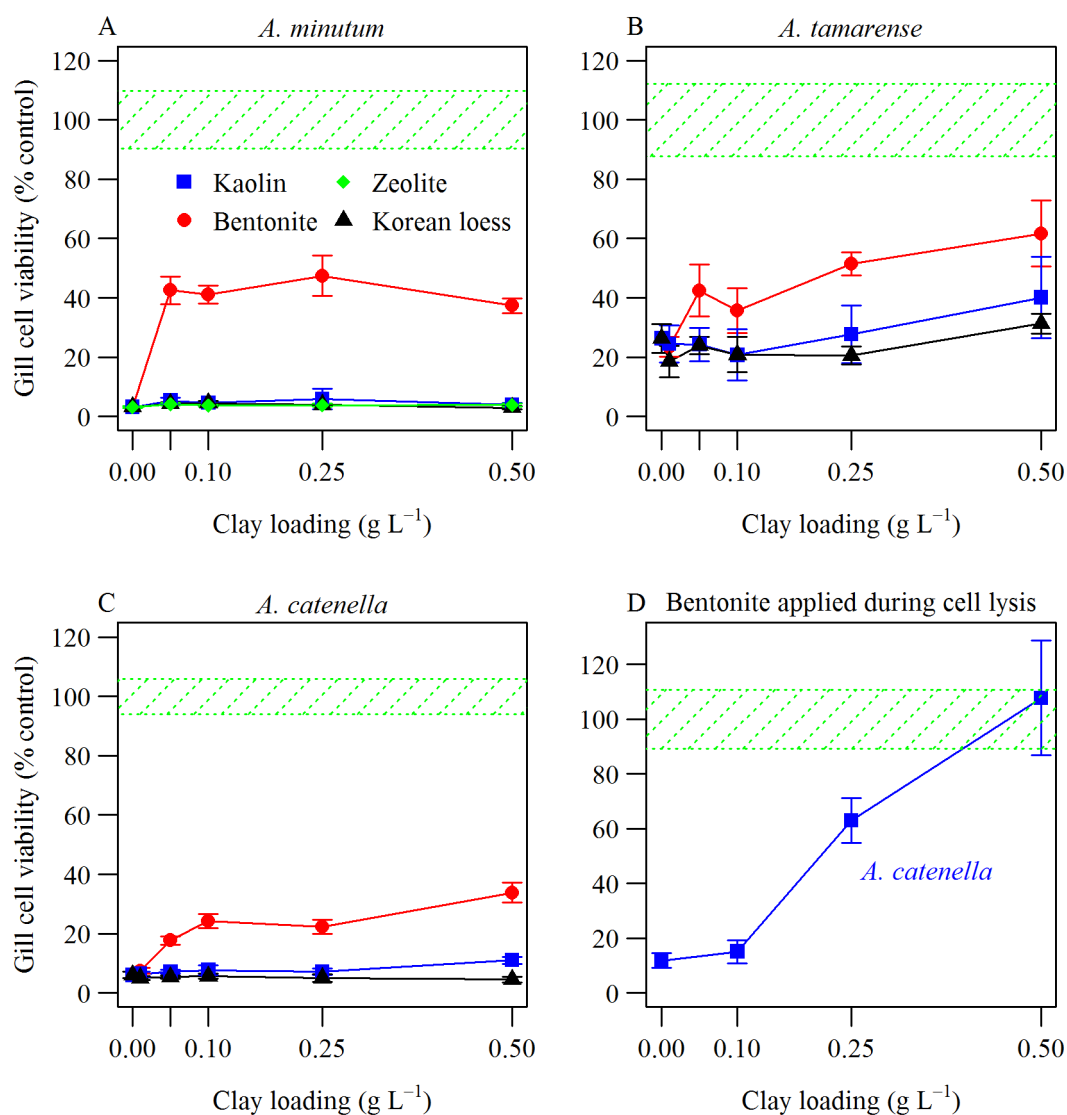


Figure 6.1 Viability of gill cells exposed to lysed preparations of *A. minutum* (A), *A. tamarensis* (B) and *A. catenella* (C) treated with 0-0.5 g L⁻¹ of kaolin, zeolite, Korean loess or bentonite clay. (D) Direct application of bentonite clay during lysis of *A. catenella* cells. The green shaded area indicates 1 standard deviation around the nontoxic control (seawater medium + treatment).

6.3.2 Mechanism of ichthyotoxin adsorption

Exposure of the gill cell line to a commercial preparation of the polyunsaturated fatty acid docohexanoic acid (DHA) at 7 µg mL⁻¹ reduced gill cell viability to 18±5% of the control (Fig. 6.2a). However, treatment of these preparations with bentonite clay completely eliminated this toxic effect at a clay loading of only 0.1 g L⁻¹.

All three *Alexandrium* species produce significant amounts of superoxide anion (O_2^-) immediately after cell lysis (Fig. 6.2b), with a one-way ANOVA proving the extent of O_2^- production to be species specific ($F_{2,6} = 76.83$, $p = 0.0001$). Total superoxide production was highest in *A. catenella* (18.2 ± 0.7 ; Tukey's HSD $p = <0.001$ when compared to both other species), followed by *A. tamarense* (12.9 ± 0.8) and *A. minutum* (11.7 ± 0.5 pmol cell⁻¹ h⁻¹; no significant difference between the latter two; Tukey's HSD $p = 0.12$). However, taking into account total cell volume (Table 6.1), *A. minutum* exhibited the highest superoxide production (2.51 ± 0.1 fmol μm^{-3} h⁻¹), followed by *A. catenella* (1.26 ± 0.05) and *A. tamarense* (0.81 ± 0.05).

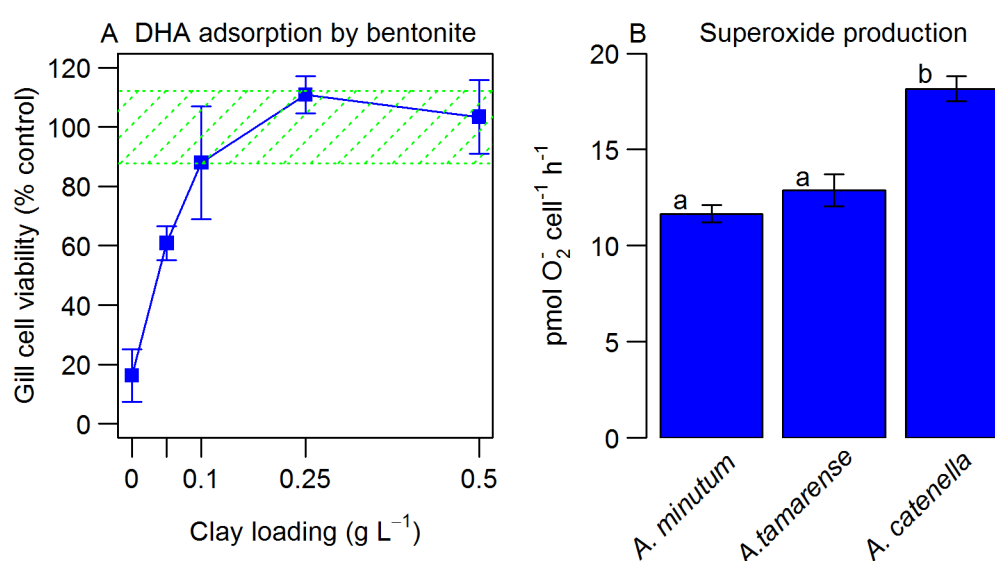


Figure 6.2 (A) Gill cell viability after exposure to a commercial preparation of docohexanoic acid (DHA, 7 $\mu\text{g mL}^{-1}$) treated with 0-0.5 g L⁻¹ bentonite clay. The green shaded area indicates 1 standard deviation around the nontoxic control (seawater medium + treatment). (B) Superoxide anion (O_2^-) production by *Alexandrium minutum*, *A. tamarense* and *A. catenella* (pmol cell⁻¹ h⁻¹) quantified within 5 min of cell lysis. Letters indicate significant differences in O_2^- production between algal species (Tukey's HSD).

6.3.3 Modified clays

Treatment of *A. catenella* cultures with pure polyaluminium chloride (PAC) or PAC modified Korean loess during cell lysis could not improve gill cell viability when compared to the untreated, toxic control (Fig. 6.3a). Only co-application of

bentonite and PAC could improve gill cell viability ($69 \pm 5.3\%$ viability at 0.25 g L^{-1}). Addition of Fe-aminoclay demonstrated no effect on gill cell viability within the concentration range assessed (Fig. 6.3b).

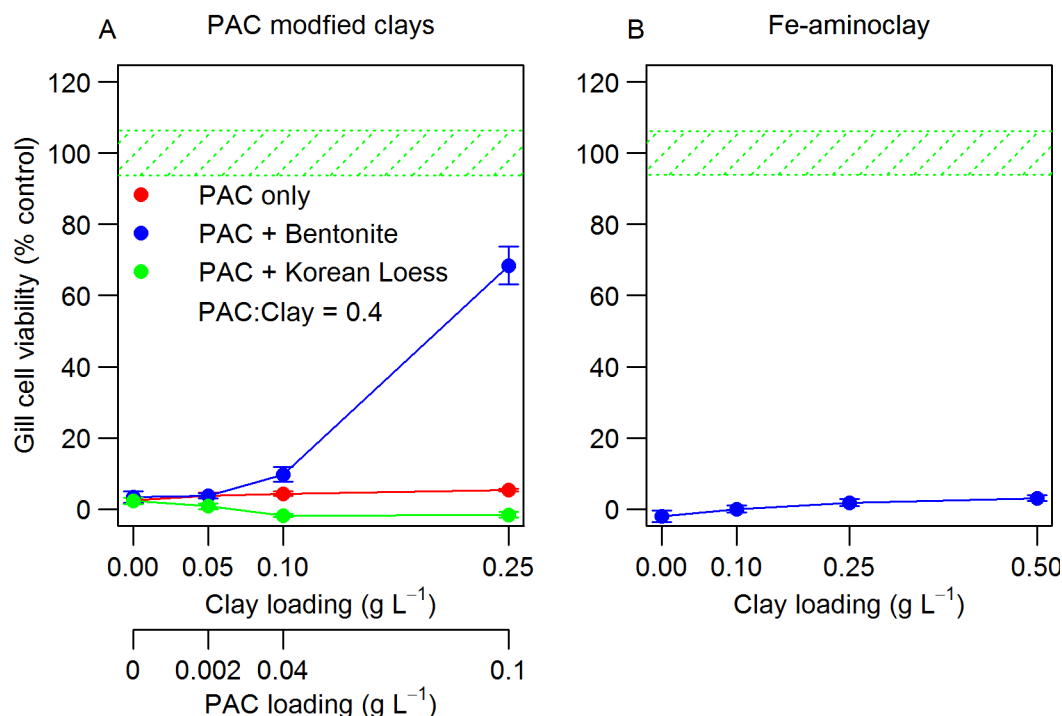


Figure 6.3 Gill cell viability after exposure to *A. catenella* preparations treated with either PAC modified bentonite, Korean loess, pure PAC (A) or Fe-aminoclay (B) during cell lysis. Pure PAC concentration was 0.4 times that indicated by clay loading. The green shaded area indicates 1 standard deviation around the nontoxic control (seawater medium + treatment).

6.4 Discussion

All three dinoflagellates *Alexandrium catenella*, *A. minutum* and *A. tamarense* proved highly toxic towards the gill cell line, but their ichthyotoxicity could be significantly reduced through treatment with bentonite clay. Striking similarities in superoxide anion production and toxin adsorption profiles between the *Alexandrium* species and raphidophyte *Chattonella marina* (Chapter 5) were observed.

The here demonstrated high ichthyotoxicity of the *Alexandrium tamarense* strain ATTR/F (20% gill cell viability after exposure to 4740 cells mL⁻¹), serves as a timely reminder of the risk that fish-killing algal blooms pose to expanding aquaculture operations. The strain was isolated from a severe bloom event in 2012 on the East Coast of Tasmania, Australia, where it is believed to previously only have occurred as a cryptic species (Hallegraeff and Bolch, 2016). Recent plans for the creation of new salmon farm leases in the area, previously only used for shellfish production (\$AUD 23M loss in 2012), pose a risk for fish mortalities.

High production of superoxide had previously only been demonstrated for *Alexandrium catenella* (Mardones et al., 2015), with Kim et al. (1999) detecting another reactive oxygen species, H₂O₂, in *A. tamarense* (no strain code supplied). We confirmed that *A. tamarense* (ATTR/F) and *A. minutum* (AMCS323) both produced significant total amounts of superoxide anion of the same order of magnitude as the well-known ROS producer *Chattonella marina* (Dorantes-Aranda et al., 2015). *Alexandrium catenella* exhibited the highest total superoxide production, but when standardised for cell volume, ROS production of considerably smaller *A. minutum* cells proved higher than that of larger *A. tamarense* and *A. catenella*. Oda et al. (1997), described similar observations when comparing several larger *C. marina* and smaller *H. akashiwo* strains. Care should be taken to not misrepresent the role of superoxide production, as the extent of lipid peroxidation is a product of cellular PUFA content and O₂⁻ generation, as well as cell density.

Similar to Chilean *A. catenella*, Scottish *A. tamarense* has been shown to produce significant amounts of docosahexanoic acid (DHA, Hammann et al., 2013). Similar to eicosapentaenoic acid (EPA) in *C. marina* (Marshall et al., 2003), DHA readily undergoes peroxidation in the presence of ROS (Bruna et al., 1989) to yield up to 9 times more toxic end-products (Mardones et al., 2015). The synergistic role of ROS and PUFAs as driving mechanism of ichthyotoxicity therefore appears not be exclusive to the well-documented fish-killing Chilean *A. catenella* strains.

If reactive oxygen species mediated lipid peroxidation were involved, very similar ichthyotoxin adsorption profiles to those described in the previous chapter for raphidophyte algae would be expected (Chapter 5). Indeed, treatment with

bentonite clay only partially removed toxicity in lysed preparations of the *Alexandrium* strains, but proved highly effective (complete removal of cytotoxicity) when applied directly during lysis of *A. catenella* cells. Paralytic Shellfish Poisoning Toxins were initially proposed to be responsible for fish kills by *Alexandrium* (Montoya et al., 1996; Sephton et al., 2007) and are produced in substantial quantities by all three strains assessed here (Table 6.1). However, any significant contribution to the observed differences in adsorption between the different clay addition scenarios is highly unlikely, due to their low contribution to overall cytotoxicity (Mardones et al., 2015) and rapid adsorption to clay minerals (Burns et al., 2009; Lu et al., 2015).

Just like EPA in case of *C. marina*, toxicity of *Alexandrium*'s major fatty acid DHA was readily removed by bentonite clay application. We suggest that similar to raphidophyte algae (Chapter 5), the direct application of clay during cell lysis removes polyunsaturated fatty acids before they can undergo lipid peroxidation to non-absorbable end-products. In the present work we only documented this for *A. catenella*, but did not test direct application of clay to *A. minutum* or *A. tamarense*. Partial removal of toxicity in these isolates could indicate the presence of other, yet uncharacterised cytotoxic compounds that cannot be removed by bentonite application.

While the comparison between the timing of clay addition (during or after cell lysis) serves to illustrate the potential effect of ROS on adsorption kinetics, bentonite type clay proved well suited for potential field applications (i.e. direct application of clay during cell lysis). As previously described for other ichthyotoxic algal species (Chapter 2-5), conventional kaolin, zeolite and Korean loess did not match the performance of bentonite clay (complete removal of *A. catenella* toxicity), even at cell concentrations that were 3 times higher ($12,000 \text{ cells mL}^{-1}$) than commonly observed in nature (Mardones et al., 2015).

Flocculation of armoured *Alexandrium* dinoflagellate cells by conventional clay minerals has been proven to be ineffective, with clay loadings of 0.5 g L^{-1} only removing 50% of *A. tamarense* cells (Sengco et al., 2001). However, the efficiency of cell removal of conventional clay can be greatly increased through the addition of

the flocculant polyaluminium chloride (PAC) or synthetic amino-clays. Both PAC and Fe-aminoclay proved effective adsorbent of *C. marina* cytotoxins (Chapter 5), but in the present work could not remove *A. catenella* toxicity towards the gill cell line. While ichthyotoxin removal efficiency of various clay types, including Korean loess and Kaolin could be greatly improved by PAC (Chapter 5), successful removal of *A. catenella* ichthyotoxicity could only be achieved with bentonite clay. For effective mitigation of fish-killing effects, it is imperative to carefully select the type of clay to be modified with PAC based on the algal species present.

6.5 Conclusions

Challenging rainbow trout gill cells with lysed preparations of *Alexandrium catenella*, *A. tamarense* and *A. minutum* proved all three species to be highly ichthyotoxic. This toxicity could be significantly reduced through bentonite clay addition and, in case of *A. catenella*, completely eliminated. Ichthyotoxin adsorption profiles for the *Alexandrium* isolates tested here, as well as high superoxide production by all three species suggest involvement of reactive oxygen species mediated lipid peroxidation. This toxicity could only be completely removed through treatment with bentonite type clay, with the flocculant polyaluminium chloride (PAC) and algicidal, synthetic Fe-aminoclay proving unsuitable. While PAC could greatly improve cell flocculation indiscriminate of clay type, the present work conclusively demonstrated that it is imperative to carefully select the correct type of clay to be modified (bentonite) to achieve complete removal of *Alexandrium* ichthyotoxicity.

Chapter 7

Conclusions:

Practical implications for aquaculture fish farm management

7.1 Introduction

The multi-million dollar global impacts of algal blooms on the finfish aquaculture industry have been well documented (Cembella et al., 2002; Hallegraeff, 1993; Imai et al., 2006; Taylor and Trainer, 2002), yet the exact chemical nature of most ichthyotoxins remains poorly characterised (Dorantes-Aranda et al., 2015; Rasmussen et al., 2016). The only fish farm mitigation strategies being widely practised include cessation of fish feeding, towing away of cages from affected areas, perimeter skirts to protect against algal surface slicks, aeration or airlift upwelling to dilute harmful algal concentrations, or clay flocculation to temporarily remove algal cells (Rensel and Whyte, 2003). Killing algal cells via chemical treatment risk cell lysis and amplification of ichthyotoxic effects through release of intracellular metabolites (Deeds et al., 2002), while unforeseeable ecological consequences make biological control with grazers (Jeong et al., 2003; Jeong et al., 2008), viruses (Nagasaki et al., 1999) or bacteria (Bai et al., 2013) unattractive and impractical for large-scale field application. Clay application, as practised in Korean and Chinese waters, offers promise as a strategy for removal of harmful algal cells, but cell lysis from physical contact between algal cells and clay particles limits its efficacy (Lee et al., 2013b; Rivera, 2014, October; Seger et al., 2015b; Seger et al., 2017; Sengco, 2001; Sengco et al., 2001; Shirota, 1989). No effective mitigation strategies currently exist that focus on removing the ichthyotoxins themselves. The present thesis work therefore explored the adsorption of ichthyotoxins from all major ichthyotoxic algal groups to a variety of clay minerals. Practical implications for aquaculture fish farm management are discussed.

7.2 Clay type and algal species specific ichthyotoxin adsorption

Screening of different clay types against a wide variety of ichthyotoxins demonstrated that clay treatment could significantly reduce cytotoxicity of all 10 fish-killing algal species assessed here. While previous work with *P. parvum* and *Karenia brevis* (Pierce et al., 2004; Sengco et al., 2005) only investigated ichthyotoxin removal by phosphatic clays, we demonstrated that ichthyotoxin adsorption extended to other clay minerals. Application success proved dependent upon the type of ichthyotoxin present (summarised in Table 7.1). Kaolin, zeolite and Korean loess all removed toxicity of select algal species to a certain extent. However, only bentonite clay consistently exhibited high adsorption across all ichthyotoxins tested, with up to 10 times lower concentrations required for complete elimination of cytotoxicity than for other clays.

Initial experiments focused on mitigating *Prymnesium parvum* ichthyotoxicity by manipulating nutrient regimes as proposed by Kurten et al. (2007) through addition of phosphate adsorbing clay (**Chapter 2**). Unexpectedly, a much greater immediate benefit from clay application was found to be the complete removal of *Prymnesium* toxicity towards the gill cell line RTgill-W1. Subsequent screening of 14 different clays belonging to the kaolin, zeolite, Korean loess and bentonite groups revealed the latter to be best suited for removal of *Prymnesium* cytotoxicity at ecologically relevant pH. Within the bentonite group, clay purity proved an important indicator, with unweathered clays originating from deeper within the clay deposits exhibiting higher ichthyotoxin removal. Flocculation efficiency of *P. parvum* cells proved to be a poor indicator of ichthyotoxin adsorption. The risk of selecting clays solely based on cell removal was convincingly illustrated in Korean field experiments (**Chapter 3**). Application of conventional Korean loess, a poor ichthyotoxin adsorbent of large particle size, effectively reduced *Cochlodinium polykrikoides* cell concentrations, but inadvertently amplified ichthyotoxicity by promoting cell lysis (US\$1.2M fish lost at Korean test site). Only when the loess was milled to achieve finer particle size (<30 µm), could complete removal of ichthyotoxicity be achieved. Reducing clay particle size not only proved effective for this species, but significantly increased karlotoxin removal, albeit only for high swelling bentonites (swell index >20 mL/2g, **Chapter 4**).

The swell index of bentonite clays serves as a general indicator of their purity, is freely available for commercial clay products and can readily be measured in the laboratory to facilitate clay selection.

Application of clay for ichthyotoxin adsorptive purposes was successfully extended to raphidophyte microalgae (**Chapter 5**). By careful consideration of their complex, reactive oxygen species mediated ichthyotoxic mechanism, it was demonstrated that bentonite clay rapidly adsorbs polyunsaturated fatty acids, such as eicosapentaenoic acid, before they can undergo lipid peroxidation. Ineffective cell removal by conventional clays of large *Chattonella marina* cells (<20% at 0.5 g L⁻¹) was greatly improved through addition of polyaluminium chloride (PAC) or synthetic aminoclays, both of which completely eliminated *C. marina* cytotoxicity. Even Korean loess, a poor adsorbent on its own, achieved complete removal of cytotoxicity when modified with PAC, indicating that ichthyotoxin removal of clay types other than bentonite could be improved. Although dinoflagellates of the genus *Alexandrium* displayed remarkably similar adsorption profiles to the raphidophyte algae for conventional clays, further experiments with *Alexandrium* dinoflagellates proved ichthyotoxin adsorption by PAC and aminoclay to be species specific (no adsorption, **Chapter 6**). To avoid inadvertent amplification of ichthyotoxic effects through clay mediated cell lysis, it is therefore of critical importance to carefully select the type of clay to be modified based on the algal species present.

In this comprehensive work, clays were challenged with a “worst-case-scenario” of complete algal cell lysis at high cell densities (up to 3 times those commonly encountered in nature) to arrive at conservative estimates of minimum effective clay dosages. Bentonite concentrations required to achieve complete elimination of cytotoxicity towards the highly sensitive gill cell line RTgill-W1 varied between algal species and their known ichthyotoxic mechanisms (0.05-0.5 g L⁻¹), but concentrations needed generally proved much lower than minimum effective concentrations required for cell removal (0.25-10 g L⁻¹, Table 7.1).

The clay concentrations used for effective ichthyotoxicity removal are orders of magnitude lower than those implicated in causing harmful effects on benthic marine invertebrates (Shumway et al., 2003).

Table 7.1 Application of clay to mitigate ichthyotoxic algal blooms. Ichthyotoxin adsorption by clay minerals of all 10 major fish-killing algal species assessed in the current work is summarised and compared to published cell flocculation studies. The effect of lysed algal preparations on gill cell viability and superoxide production is indicated. Where no reference is given, values represent the current work.

Species	Ichthyotoxicity			Ichthyotoxin adsorption			Cell flocculation		
	% gill cell viability	Concentration (Cells mL ⁻¹)	Superoxide production (pmol cell ⁻¹ h ⁻¹)	Ichthyotoxin (%)	Clay type	Clay concentration (g L ⁻¹)	Cell (%)	Clay type	Clay concentration (g L ⁻¹)
Haptophytes (Chapter 2)									
<i>Prymnesium</i>	40	1 x 10 ⁵	Negligible ^a	100	Bentonite	0.25	100	Bentonite	1
				0	Kaolin	1	100	Korean loess	1
				0	Korean loess	1	95 ^b	Bentonite + PAC	0.5
				0	Zeolite	1			
Unarmoured dinoflagellates (Chapter 3 & 4)									
<i>Cochlodinium polykrikoides</i>	10 80	1.4 x 10 ⁴ 1.1 x 10 ³	22	100	Milled loess	0.5	60	Korean loess	0.5
				0	Conventional loess	0.5	80 ^c	Fe- and Ca-aminoclays	0.1
							95 ^d	Korean loess +sophorolipid	1
<i>Karenia mikimotoi</i>	60	1.1 x 10 ⁵	0.99-3.29 ^a	100	Bentonite	0.05	N/A**		
				100	Kaolin	0.5			
				0	Korean loess	0.5			
				0	Zeolite	0.5			
<i>Karlodinium veneticum</i>	5	1.1 x 10 ⁵	0.06-0.49 ^a	100	Bentonite	0.1	80	Bentonite	0.5
				0	Kaolin	0.5			
				0	Korean loess	0.5			
				0	Zeolite	0.5			

<i>Karenia brevis</i>	0-45% for brevetoxins ^a PBTX-2 and -3		-	67-75	Bentonite	0.25	90-95 ^e	Bentonite	0.25-0.5
				100	Bentonite + PAC	0.25	85 ^f	Bentonite + PAC	0.25
Raphidophytes (Chapter 5)									
<i>Chattonella marina</i>	20	1.5 x 10 ³	2.48-14.03 ^a	100	Bentonite	0.25	20	Bentonite	0.5
				0	Kaolin	0.5	90	Bentonite + PAC	0.1
				0	Korean loess	0.5	12 ^g	Kaolin	0.4
				100	PAC	0.1	100 ^g	Vermiculite + Gemini surfactant	0.065
				100	Korean loess +PAC	0.25		Fe-aminoclay	0.1
				100	Fe-aminoclay	0.25	100	Bentonite	0.25
<i>Heterosigma akashiwo</i>	5	9 x 10 ⁴	0.13-0.40 ^a	100	Bentonite	0.25	90 ^h	Bentonite	0.25
				20	Kaolin	0.5	100 ^c	Ca-aminoclay	0.05
				20	Korean loess	0.5			
				20	Zeolite	0.5			
<i>Fibrocapsa japonica</i>	60	6 x 10 ⁴	0.01-1.94 ^a	20	Bentonite*	0.5			
				20	Kaolin	0.5			
				10	Korean loess	0.5			
				5	Zeolite	0.5			
Armoured dinoflagellates (Chapter 6)									
<i>Alexandrium catenella</i>	0	4.7 x 10 ²	18	100	Bentonite	0.5			
				0	Kaolin	0.5			
				0	Korean loess	0.5			
<i>Alexandrium tamarense</i>	20	2.7 x 10 ²	13	20	Bentonite*	0.5	40 ^h	Bentonite	4
				10	Kaolin	0.5	84 ⁱ	Korean loess	10
				20	Korean loess	0.5	100 ^j	Kaolin + PAC	0.25
<i>Alexandrium minutum</i>	0	2.7 x 10 ³	12	20	Bentonite*	0.5			
				0	Kaolin	0.5			
				0	Korean loess	0.5			
				0	Zeolite	0.5			

Commercial ichthyotoxin preparations (Chapter 5 & 6)						
Polyunsaturated fatty acids						
Fresh EPA	4	50 µg.mL ⁻¹	-	100	Bentonite	0.5
Stored EPA	17	5 µg.mL ⁻¹	-	20	Bentonite	0.5
Fresh DHA	18	7 µg.mL ⁻¹	-	100	Bentonite	0.5
Fatty acid aldehydes						
Heptadienal	39	15 µg.mL ⁻¹	-	0	Bentonite	0.5
Decadienal	62	3 µg.mL ⁻¹	-	0	Bentonite	0.5

* Prediction based on other species within group. See Chapters 4 (Raphidophytes) and 5 for details (*Alexandrium* spp.).

** No records found in literature.

^a (Dorantes-Aranda et al., 2015)

^b (Sengco et al., 2005)

^c (Lee et al., 2013b)

^d (Sun et al., 2004b)

^e (Pierce et al., 2004)

^f (Sengco and Anderson, 2004)

^g (Wu et al., 2010)

^h (Sengco et al., 2001)

ⁱ (Sun and Choi, 2004)

^j (Lu et al., 2015)

7.3 Targeted approach to clay application

In Korean waters, 1000-97,000 tons of clay are applied each year (Park et al., 2013) and repeated concerns have been raised for impacts on the benthos from the high loads of suspended particles and sedimentation of toxic algal detritus (Archambault et al., 2004; Beaulieu et al., 2005; Shumway et al., 2003). Lewis et al. (2003) and Haubois et al. (2007) demonstrated that clay flocculation of *Karenia brevis* cells did not increase toxicity to benthic organisms compared to the effect from an untreated bloom. The economic imperative to improve cell and ichthyotoxin removal and at the same time reduce clay loadings is significant. Rather than creating a frontal clay plume barrier against approaching algal blooms as practised in South Korea, we propose a more targeted application of clay designed to not only maximise cell flocculation but taking into account the ichthyotoxin adsorptive properties of clay. Fish from mass mortality events have previously been dumped offshore (Chile, March 2016), in landfills (Australia, 1996) or processed into fertiliser, yet no human health effects by ichthyotoxins have been reported (with the exception of *Karenia brevis* and related species). Our targeted approach aims to provide temporary relief of fish-killing effects to localised aquaculture resources to allow for equipment to be put in place for emergency harvesting of often permanently compromised finfish.

The two clay dispersal strategies of broad cell flocculation and emergency harvest preparation differ considerably in their spatial scales, as well as personnel requirements, clay loading and total cost involved (summarised in table 7.2). To maximise cell, as well as ichthyotoxin removal, we recommend the use of PAC modified, high-swelling bentonites. PAC was selected from the numerous clay modifiers available (Sengco et al., 2001; Sun et al., 2004b; Wu et al., 2010), as it has passed extensive risk assessments due to its wide use in drinking water treatment throughout the world (Srinivasan et al., 1999). Bentonite clay is considerably more expensive than Korean loess (\$US800 and \$US18, respectively), but its application

can be made economically feasible (\$US4000-6000 per day) due to much lower clay loading required, and much shorter time and smaller spatial scale of application.

Table 7.2 Comparison between large scale frontal barrier approach to clay dispersal practised in Korean waters and the here proposed fish pen targeted application regime.

Approach	Frontal barrier (Korea)	Targeted*
Area treated	200,000 km ²	1.5 km ²
Dispersal	Constant	5 times day ⁻¹
Application period	25-50 days	7 days
Ship(s)	20-40 = \$US60,000 day ⁻¹	1 = \$US1,500 day ⁻¹
Labor	\$100 day ⁻¹ person ⁻¹ x 50-100 people = \$5000-10000 day ⁻¹	\$500 day ⁻¹ x 4 people = \$2000 day ⁻¹
Clay	Conventional Korean loess \$18 ton ⁻¹	Bentonite: \$800 ton ⁻¹ PAC: \$300 ton ⁻¹
Clay loading	400 g m ⁻²	100-400 g m ⁻²
To treat area	Korean loess: 80 tons	Bentonite: 0.15-0.6 tons PAC: 0.06-0.24 tons
Total cost per day	US\$36,000 - 72,000	\$4,000-6,000

* Cost estimates modified from Anderson et al. (2001) for ichthyotoxin adsorptive purposes.

The proposed application area of 1.5 km² is based on 20 average sized fish pens (25 m²) and takes into account a safety radius 3 times the total cage area. It should be noted that clay loadings and application regimes are indicative only, based on the present work and information provided in Table 7.1. Adaptation to local, site specific conditions, such as current flow (Archambault et al., 2003) and density of harmful algal cells (Sengco et al., 2005) will be required to achieve maximum treatment efficacy and effective mitigation of fish-killing effects.

7.4 Recommendations for future research

Clay loadings and application regimes will need to be adapted to local, site specific conditions that cannot easily be replicated in the laboratory. Further refinement of clay application therefore will require field trials to provide fish farmers with a cost-effective emergency response to mitigate fish-killing effects of harmful algal blooms.

Modification of conventional clays with polyaluminium chloride demonstrated that otherwise ineffective adsorbents, such as Korean loess, can be rendered highly

effective against *C. marina* ichthyotoxins and replace more expensive bentonite clay. While this promises much reduced application costs, the effectiveness of PAC modified clays against other algal species and their ichthyotoxic mechanisms remains to be clarified.

A recent outbreak (2016) of the dictyophyte *Pseudochattonella* in Chilean fjords led to social unrests due to catastrophic salmon mortalities. Clay application for ichthyotoxin adsorptive purposes was demonstrated in this thesis to be effective against a wide range of ichthyotoxic algal species, but remains to be investigated for fish-killing dictyochophyte microalgae.

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